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PRODUCTION OF ANTIBIOTICS BY FUNGI. PART II: PRODUCTION BY *FUSARIUM JAVANICUM* AND OTHER *FUSARIA*.

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THE production of antibiotic metabolism solutions by a miscellany of fungi under varying culture conditions was described in a previous paper (Cook and Lacey, 1945). The present paper records the results of a similar study of 19 species and strains of *Fusarium*, and a more detailed examination of the behaviour of *F. javanicum* and the antibiotic pigment, javanicin, produced by it.

EXPERIMENTAL.

Methods.

In general, the methods and media employed for the examination of *Fusarium* culture fluids for antibacterial activity were similar to those described by Cook and Lacey (1945). It was found, however, that the agar plate method of testing did not give a reliable quantitative estimation of the antibacterial titre of javanicin solutions. In an acid medium javanicin diffused freely into the agar, producing clear zones of inhibition, but in neutral or alkaline solutions little or no diffusion occurred. The serial broth dilution method was therefore employed throughout in testing javanicin solutions for antibacterial activity.

Results with Species other than F. javanicum.

(*a*) Negative results were obtained in all tests of cultures of the following: *F. culmorum* (one strain), *F. caeruleum*, *F. oxysporum cubense* and 4 unidentified species. In addition a strain of *F. dianthi* and an unidentified species developed only a trace of activity, and this only irregularly.

(*b*) The culture fluid of a second strain of *F. culmorum* grown on a medium containing 1 per cent Bacto-Tryptone and 4 per cent glucose was inactive against *Staphylococcus aureus*, but was both bacteriostatic and bactericidal towards *Myco. phlei* in 1 : 10 dilution from the 6th to the 15th day; the activity had disappeared by the 19th day.

(*c*) Culture solutions of *F. fructigenum* were always inactive towards *Staph.*

aureus. No activity towards *Myco. phlei* developed in media containing 1 per cent Bacto-Tryptone without glucose, but media containing 1 per cent glucose became bacteriostatic and bactericidal (dilution 1 : 10) towards *Myco. phlei* between the 13th and 17th days ; in 2 or 4 per cent glucose activity was delayed until the 20th day, the solutions being bacteriostatic (1 : 10) but not bactericidal.

(*d*) Three strains, F 71 and F 75 (? *F. lateritium*) and F. 80 (*F. avenaceum*) grown in a medium containing 1 per cent Bacto-Tryptone and 4 per cent glucose caused complete inhibition (1 : 10) of *Staph. aureus* and (1 : 20) of *Myco. phlei* from the 13th day onwards.

(*e*) *F. sambucinum* was examined at intervals over 18 months in a variety of media. In synthetic media containing 0·5–4 per cent glucose and supplemented with varying amounts of yeast extract, feeble activities towards *Staph. aureus* and *Str. pyogenes* and stronger activities towards *Myco. phlei* were developed but growth of *Bact. coli* was unaffected. Results were similar when yeast extract was replaced by 0·5 per cent Bacto-Tryptone. In media containing 1 per cent Bacto-Tryptone, 0, 0·5, 2 or 4 per cent glucose, but no added salts, plate tests showed very feeble activity towards *Staph. aureus* and *Str. pyogenes* (1 : 5–1 : 10), with stronger activity towards *Myco. phlei* (1 : 25) in all cultures alike. Dilutions of 1 : 10 were bactericidal towards *Myco. phlei*. The last titres were reached on the 6th day, and persisted for 6 weeks.

(*f*) An unidentified *Fusarium* (F 6) grown in 1 per cent glucose and 1 per cent Bacto-Tryptone produced activity similar to that developed by *F. fructigenum* (+ towards *Myco. phlei*, — towards *Staph. aureus*). It differed from *F. fructigenum* however in developing activity towards both organisms in Bacto-Tryptone media containing 4 per cent glucose ; 1 : 10 dilutions were bactericidal towards both from the 6th until the 17th day, when the last tests were made. Activity of the last kind was also shown by another unidentified *Fusarium* (F. 72). Still another strain (F. 64) produced complete inhibition of *Staph. aureus* and *Myco. phlei* in 1 : 5 dilution at 12–15 days on a medium containing 1 per cent Bacto-Tryptone and 4 per cent glucose. The solutions were bactericidal towards *Myco. phlei* on the 12th day, but only bacteriostatic by the 19th day. In this instance, but not in others, Bacto-Tryptone could be replaced by Eupepton.

The Production of Antibiotics by F. javanicum.

It was earlier reported (Cook and Lacey, 1945) that of several *Fusarium* cultures *F. javanicum* was the most potent in developing antibiotic solutions. Many of the 18 strains used in the preceding section exhibited pink, orange or yellow mycelia, but the pigments did not diffuse into the medium and appeared to have no connection with the antibacterial activity of the solution (Sideris, 1925). Strains of *F. javanicum* differed, however, from the preceding ones in producing, under selected cultural conditions, a blood-red pigment which diffused into the medium (liquid or solid) and was found to be the active antibacterial principle. The pigment fraction was sometimes substantially homogeneous, but at other times contained two antibacterial pigments. These have been termed javanicin, $C_{15}H_{14}O_6$, the major constituent, and oxyjavanicin, $C_{15}H_{14}O_7$. Their preparation and chemistry is described elsewhere (Arnstein, Cook and Lacey, 1946 ; Arnstein and Cook, in press), and the present account is restricted to considerations of biological interest.

Cultural conditions.

Addition of Bacto-Tryptone or Eupepton to the medium was found essential for production of antibacterial activity so that these nutrients in synthetic medium containing 4 per cent glucose could not be replaced by yeast extract (three preparations were examined), 1 per cent malt extract, 2 per cent concentrated corn-steep liquor, 1 per cent. meat extract (two preparations) or 1 per cent Gurr's peptone.

When glycine (0·05, 0·1, 0·2 and 0·4 per cent) was substituted for Bacto-Tryptone, pigmentation appeared to be equally good, but the activity of the medium was much less (about 1 : 5 after 17 days). The effect of glycine at the various levels was compared by extracting the metabolism solutions with ether, removing inactive pigment with sodium bicarbonate, and taking the residue to dryness. The solid residue was then dissolved in ethanol and the colours compared visually. It was found that 0·5 per cent glycine was the optimum concentration. Although no solid javanicin was isolated from this medium there can be no doubt that the colour was due to javanicin, for the absorption spectrum of this solution was almost identical with that of pure javanicin. Brown and Horne (1926) found that the addition of ammonium chloride increased the amount of yellow pigmentation of certain species of *Fusaria*. When ammonium chloride (0·05, 0·1 and 0·2 per cent) was substituted for Bacto-Tryptone the mycelium of *F. javanicum* was pigmented red, but although growth appeared otherwise normal, there was no diffusible pigment of any kind. The activity of these media was not tested.

Four other preparations were tested as substitutes for Bacto-Tryptone and Eupepton. The effect of these materials is shown in Table I, none being a useful substitute.

TABLE I.—*Effect of Various Preparations on the Production of Antibiotic by* F. javanicum.

	Time of incubation (days).	Antibiotic activity against *Staph. aureus*. (Dilution of culture fluid.)
Glycine (0·05 per cent)	12	1 : 5
	17	1 : 5
Eupepton (0·5 per cent)	12	1 : 50
	14	1 : 100
Vitamin-free acid digest of casein (0·5 per cent)	15	1 : 25
Low ash casein digest (0·5 per cent)	8	1 : 25
	15	1 : 50 to 1 : 100
Meat protein papain digest (0·5 per cent)	8	1 : 10
	15	1 : 25
Baker's peptone (0·5 per cent)	13	1 : 10

Deep red pigmentation diffusing into the medium and an activity titre of 1 : 100 towards *Staph. aureus* and *Myco. phlei* was produced in synthetic media containing 4 per cent glucose and 0·5 per cent Bacto-Tryptone. Smaller

amounts of glucose (1 per cent) caused earlier development of activity and pigment (observable after 3 days), but production soon ceased and the maximum activity titre was lower. Without glucose there was no development of either pigment or activity.

The effect of varying the initial pH was investigated using a Czapek-Dox medium containing 4 per cent glucose and 0·5 per cent Eupepton. At pH 4 the fungus grew very slowly with slow development of pigment and activity. At pH 5 this development was more rapid and was maximum at pH 6. Brownish coloration of the fluid and green mycelia were observed in cultures initially at pH 7, and these effects were enhanced at pH 8. At pH 7–8, however, only a trace of anti-staphylococcal activity developed. The optimum pH was slightly higher (6·8) in Bacto-Tryptone media.

The optimum temperature of incubation was about 25° C. At 22° C. growth and pigment production were slower, though the ultimate maximum titre was the same. At 30° C. rate of growth and antibiotic production as well as the absolute amount of antibacterial activity were diminished, and growth failed at 37° C.

Although the antibacterial effect was most readily observed in the culture fluid, considerable quantities of javanicin were present in the mycelia. Thus a 15-day culture in Bacto-Tryptone-glucose broth (40 ml., anti-staphylococcal titre 1 : 80) was drained and the felt ground with acetone (20 ml.). The extract completely inhibited the growth of *Staph. aureus* at a dilution of 1 : 400–1 : 500 and of *Myco. phlei* at 1 : 800.

Variation of pigment production with strain.

Colonies of *F. javanicum* grown on Bacto-Tryptone or Eupepton glucose agar media became deep red, the pigment being at first observed within the hyphae and gradually diffusing into the medium until the whole plate was stained blood-red. The red pigment was also produced slowly and less strongly on malt extract agar, but on other media such as potato-agar the cultures remained white. On a synthetic medium containing 3 per cent glucose the deep mycelial coloration as noted above in liquid cultures at pH 8 was observed, the solid medium itself being stained yellow. Both green and yellow pigmented extracts were inactive towards *Staph. aureus*.

Ten cultures on Bacto-Tryptone broth which were 4–8 months old and in which the fungal growth had degenerated into a jelly-like mass were subcultured on to a synthetic medium containing 3 per cent glucose and on to a Eupepton-glucose-agar medium ; two cultures produced no pigment under these conditions, but the remaining eight behaved in the original manner ; they produced green pigment on the former medium and red diffusing antibacterial pigment on the latter.

Single spore cultures of *F. javanicum* showed a marked variation in antibiotic production. On Bacto-Tryptone-glucose-agar the antibiotic pigmentation ranged from blood-red, orange-red and orange to almost colourless. Some colonies showed definite sectors of blood-red and white forms, the former being of markedly more restricted growth. By continued selection of deeply pigmented colonies a culture was obtained which produced nearly twice as much javanicin as the original culture.

Antibiotic activity of javanicin.

When *F. javanicum* was grown in conical flasks (150 ml.) containing Bacto-Tryptone-glucose medium the crude culture fluids normally developed a bacteriostatic titre of 1 : 200 towards *Myco. phlei* and 1 : 100 towards *Staph. aureus*, *B. subtilis* and the Gram-positive plant parasite *Bact. fascians*. The culture fluids were less potent towards *Str. pyogenes*, very feebly active towards *Bact. coli* and inactive towards *Ps. pyocyanea*, *Ps. fluorescens liquefaciens* and the Gram-negative plant parasite *Bact. tumefaciens*. After extracting pigments (Arnstein, Cook and Lacey, 1946; Arnstein and Cook, in press) the residual liquid was biologically inactive. The extracted pigments ultimately yielded javanicin and oxyjavanicin, both being antibacterial naphthoquinones. Table II shows the antibacterial activity of javanicin. We have to thank Dr. A. T. Fuller, National Institute of Medical Research, London, for tests on the haemolytic streptococcus and *Clostridium welchii*; these inhibitory effects were observed in broth. In view of the powerful action towards *Myco. phlei* its action towards *Myco. tuberculosis* was examined by Prof. W. H. Tytler, Welsh National School of Medicine, Cardiff, to whom our thanks are also due.

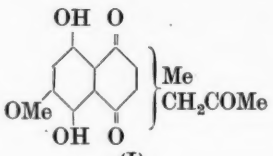
In in-vivo tests mice tolerated a maximum dose of 10 mg. (intraperitoneal injection). The bacteriostatic effect towards *Myco. tuberculosis* was not markedly affected, if at all, by serum though there was no definite evidence of a bactericidal action; however, in further tests in blood media javanicin produced a brown colour (probably methaemoglobin) at 1 : 50,000 and failed to inhibit the growth of the haemolytic streptococcus at 1 : 20,000, so that it seemed to have serious defects as a drug for systemic treatment. Prof. W. H. Tytler kindly carried out tests in which 8 guinea-pigs were inoculated intracutaneously, each on both flanks, with *Myco. tuberculosis*, and one of the resulting ulcers in each animal treated daily for 3 weeks with a 1 : 500 solution of javanicin, in N/50 alkali. On application there was a rapid colour change and precipitation, so that the effective

TABLE II.—*Antibacterial Activity of Javanicin.*

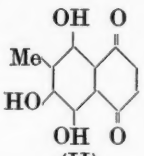
Bacterium.	Effective concentration for—	
	Bacteriostasis.	Bactericidal action.
Staph. aureus	1 : 200,000	1 : 50,000
Myco. phlei (1st strain)	1 : 200,000	1 : 40,000
,, ,, (2nd strain)	1 : 100,000	..
B. subtilis	1 : 200,000	1 : 50,000
Str. pyogenes	1 : 40,000	>1 : 40,000
Bact. fascians	1 : 100,000	..
Bact. coli	>1 : 10,000	..
Ps. pyocyanea	,,	..
B. fluorescens liquefaciens	,,	..
Bact. tumefaciens	,,	..
Myco. tuberculosis (human)	1 : 50,000 to 1 : 100,000	..
Haemolytic streptococcus	1 : 100,000	..
Cl. welchii	1 : 100,000	..

concentration of residual javanicin may have been very low or indeed negligible. There was at most a slight indication that the treated ulcers did not progress as rapidly as the untreated ones, but there was no suggestion of healing.

Javanicin (I) is a naphthoquinone related structurally to hydroxydroserone (II), and appears to owe its antibacterial properties in large measure to its



(I)



(II)

quinone nature. Thus the action of javanicin was inhibited by thiols though with difficulty as the behaviour with penicillamine shows :

Test solution. (mg./ml.) Javanicin + Penicillamine.	Limiting diluting towards *Staph. aureus*.
1·15 mg. . .	1 : 100,000–1 : 200,000
,, 0·5 .	1 : 100,000–1 : 200,000
,, 1·0 .	1 : 50,000 –1 : 100,000
,, 2·0 .	1 : 25,000 –1 : 50,000
,, 4·0 .	1 : 100,000
,, 8·0 .	1 : 50,000 –1 : 100,000

The higher figures at greater concentrations of penicillamine reflect the known but small antibacterial action of penicillamine itself.

Structural features other than the quinone grouping appear to have an influence as the smaller activity of hydroxydroserone, especially towards *Myco. phlei*, shows :

	Staph. aureus.	*Mycobact. phlei.*
Javanicin	1 : 200,000 .	1 : 200,000
Hydroxydroserone . . .	1 : 50,000 .	1 : 10,000 (44 hrs.) or inactive (3 days).

Oxyjavanicin inhibited the growth of *Staph. aureus* at 1 : 200,000, and a similar degree of activity was shown by certain derivatives of javanicin retaining the quinone system such as anhydrojavanicin and the acetyl derivative thereof.

Antifungal action of F. javanicum.

When malt-Bacto-Tryptone- or Eupepton-agar plates were inoculated with *F. javanicum* and at another point with *Verticillium albo-atrium*, *V. dahliae*, *Trichoderma* sp., *Phoma* sp., or other *Fusarium* sp., *F. javanicum* had no action on the other fungi and was itself slightly inhibited by *Trichoderma*. When, however, *F. javanicum* was first grown on Bacto-Tryptone-agar until javanicin was being freely produced and the plate then inoculated with the above fungi, zones of inhibition limited by the coloured circles of javanicin were observed.

Malt-agar plates were impregnated with a crude culture filtrate of *F. javanicum* (active against *Staph. aureus* at 1 : 50) in 1 : 10 dilution and the plates then sown with *Aspergillus parasiticus*, *Verticillium albo-atrium*, *V. dahliae*, *Trichoderma* sp., *Phoma* sp., *Penicillium notatum*, *Fusarium sambucinum*, *F. lateritium* or *F. javanicum*. The first five fungi were unaffected, but growth of *P. notatum* and the *Fusarium* species was reduced without being entirely suppressed; after 3 days, growth was about 30 per cent of the controls. A weak but specialized effect was thus apparent.

Effect of javanicin on seed germination.

In 1 : 100,000 or 1 : 50,000 concentration javanicin had no effect on germination of turnip or lettuce seeds. At a concentration of 1 : 20,000 germination of lettuce seeds was entirely suppressed, whilst with turnip seeds 60 per cent of the radicles emerged compared with 100 per cent in control solutions, but no further growth occurred. Root inhibition was still more marked in the case of tomato seeds in javanicin at a concentration of 1 : 20,000. Whereas 18 control seeds germinated and developed normally, 7 out of 18 treated seeds failed to germinate, and in the remaining 11 there was delayed emergence and growth of the plumule and complete absence of any root growth.

SUMMARY.

Twelve of 19 species and strains of *Fusarium* developed antibacterial culture solutions under selected conditions. There is evidence for the development of a plurality of antibacterial substances, some of which have been isolated.

The production of two antibacterial pigments, javanicin and oxyjavanicin, by selected strains of *F. javanicum* under a variety of conditions has been examined.

The antibacterial action of javanicin (including its in-vitro and possible in-vivo antitubercular action) and of some nearly related compounds was studied.

Javanicin had a weak specialized antifungal action and a marked effect on the germination of certain seeds.

We thank Sir Ian Heilbron, D.S.O., F.R.S., for his interest and encouragement, and the Rockefeller Foundation and Medical Research Council for financial assistance. We also thank Dr. L. E. Hawker (Imperial College), Miss J. Moore (Rothamsted Experimental Station), Mr. M. H. Moore (East Malling Research Station) and Miss E. M. Wakefield (Kew) for various cultures.

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NOTE ON A VARIANT OF *B. ANTHRACIS* WHICH FAILS TO FORM HEAT-RESISTING ENDOSPORES, AND ON ATTEMPTS TO IMMUNIZE GUINEA-PIGS AGAINST ANTHRAX INFECTION WITH KILLED VACCINES PREPARED FROM IT.

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THE fact and means of the isolation from the typical anthrax strain " Vollum " of what by way of label I have called the " Hampstead " races of that strain have been stated elsewhere (White, 1946) in describing mucoid cultures derived from them. Of the origin of these variants, of which the most striking deviation from the classic type is their failure to form thermo-resistant spores, it need only be said that they were isolated from platings of " Vollum " cultures which had been exposed to complement-activated anti-anthrax serum.

DESCRIPTION OF THE VARIANT RACES.

The variant races differ culturally from the parent strain in forming on agar after 24 hours' incubation at 37° C. colonies which, though anthracoid in type, are smaller, more raised and moistly caseous, less brambled of surface and less marginally irregular than are those of the latter (Fig. 1 and 2). Microscopically the units and chains of the young culture, though showing in general the squared-off ends and bamboo aspect characteristic of the species, fail to present the classic picture in entirety, resembling rather the bacillary relicts of an anthrax culture in which sporulation has run its course (Fig. 3, 4 and 5). The units are often scattered or in pairs, and chains tend to be short.

Capsulation.

As in the parent strain, generalized capsulation is restricted to growth in the animal body, in serous media, or in an atmosphere rich in CO_2. (In passing it may be noted that, at variance with the categoric statements in some text-books, the two typical strains of *B. anthracis* which I have studied—the Vollum strain and a strain isolated from human anthrax by Mr. P. Church at the Norfolk and Norwich General Hospital—have, when cultivated under " usual " conditions on " ordinary " media, consistently shown, as do the variants under discussion, sporadic capsulated elements in varying number.)

Sporulation.

Spores more resistant to heat or *markedly* more tenacious of life than are the vegetative rods have never been detected. All cultures are sterilized by moist heat at 60° C. in 20 minutes, are readily killed by toluol, phenol, alcohol or chloroform, and die out in a few weeks if left neglected on agar at room temperature.

Nevertheless, processes of abortive or abnormal sporulation are discernible in the formation of non-staining vacuole-like bodies about granular condensations of the stain-avid substance of the cells. Many of these bodies die and disintegrate with the bacilli in which they are produced, but others, continuing to grow within the distended parent cells, give rise to the globular elements which become a feature of the microscopic picture by the second or third day of incubation (Fig. 6) and form, with smaller grains situate in the more normal rods, the last refuge of vitality in the culture moribund with age. On transfer to fresh medium germination occurs within the globes; in each the central granule grows and segments to form a close ring of small elements (Fig. 7) which, continuing to increase as the parental wall dissolves, is liberated as a convoluted skein of short bacilli from which the culture is regenerated. Within the rods of the transplant this process of bacterial vivipary is taken up by the smaller grains to which reference has been made, and these growing at right angles to the parent axis produce the appearances seen in Fig. 8. It is doubtful whether in the aged culture growth and multiplication are ever resumed by extension and division of the original axis.

The process is curiously modified when the variant culture is grown on Sabouraud's medium. On this, in the course of a few days' incubation, the chains first formed become packed with non-staining inclusions which, on disruption of the bacilli, are shed as an assortment of hyaline spheres, often greatly exceeding in diameter the width of the parent rod, and each containing a central granule, which germinates on transfer to a favourable substrate.

Some attempts have been made to induce the Hampstead variant to resume normal sporulation by cultivating it in broth culture filtrates of the parent Vollum strain, but without success. From animal passage the cultures emerge unchanged in their peculiarity.

Virulence.

When first isolated the variant cultures showed a measure of virulence not vastly less than that of the parent strain: guinea-pigs inoculated with 1/5000th and 1/10,000th c.c of an 18 hours' broth culture died in 3–5 days with all the appearances typical of anthrax. During repeated subculture on artificial media, however, the virulence for the guinea-pig seems to have abated, and doses as high as 1/100th and 1/50th c.c. subcutaneously injected may set up a modified form of the disease in which the animal dies with massive oedema progressing from the site of inoculation, but without the usual enlargement, softening and massive infection of the spleen. This decline in virulence may at least in part be reversed by animal passage, but no attempt has yet been made to achieve a complete restoration of its pathogenic intensity.

IMMUNIZATION EXPERIMENTS.

The isolation of anthrax cultures—capsulated *in vivo* and virulent in token of their authenticity—which could be killed by methods commonly applied to the sterilization of bacterial vaccines raised the hope that they might be used for the effective immunization of laboratory animals against infection by the parent strain.

Technical Details.

Since the animal accommodation and disinfection facilities of the isolation unit available for such trials were limited, the guinea-pig was chosen as the most suitable test animal, and rabbits have been rarely used.

To supply a stock infective material for the challenge of immunity the Vollum strain, a typical representative of its kind, received in the dry state from Dr. D. W. Henderson, was seeded in broth, and when actively sporing the culture was distributed in 0·1 c.c. amounts into ampoules and in these dried over P_2O_5 *in vacuo*. As required the contents of an ampoule were taken up in 1 c.c. of broth, and 0·1 c.c. of the resulting suspension was inoculated into 5 c.c. of broth to give, after 18 to 20 hours' incubation at 37° C., the culture to be injected. The infecting dose was registered as the volume of this culture, suitably diluted with broth, which was inoculated.

Of the virulence of such cultures it may be said that 1/5000th c.c. subcutaneously injected was invariably fatal to normal guinea-pigs, that all those animals (6) which received 1/10,000th or 1/20,000th c.c. also died, and that of two animals each receiving 1/100,000th c.c. one succumbed and one survived—the only normal guinea-pig observed to survive puncture with a needle infected with this organism. When the infecting dose was in the region of 1/200–1/100th c.c. death occurred in 48 hours or less; when 1/5000th c.c. life was usually prolonged to between 3 and 5 days.

Experimental.

Preliminary experiments.

In the first place experiments were made in which guinea-pigs were intensively immunized by the subcutaneous and intraperitoneal routes, and during a month or more with heavy suspensions of the Hampstead variant cultures grown on ordinary agar media and killed by alcohol or chloroform or by heating at 60° C. The challenge dose of Vollum culture adopted was relatively small—1/5000th to 1/2000th c.c. of the "standard" broth culture. In some of the experiments one-third to two-thirds of the immunized animals survived, and in general there was prolongation of the average life of the remainder beyond that

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DESCRIPTION OF PLATE.

FIG. 1.—Photograph by obliquely transmitted light of normal colonies of *B. anthracis* (strain Vollum) from 18 hours' culture on agar. × 4½.

FIG. 2.—Photograph by same light as in Fig. 1 of colonies of Hampstead variant of the Vollum strain. 18 hours' culture on agar. × 4½.

FIG. 3 and 4.—Sporulation at 18 hours and 36 hours in an agar slope culture of *B. anthracis* (strain Vollum). × 1250.

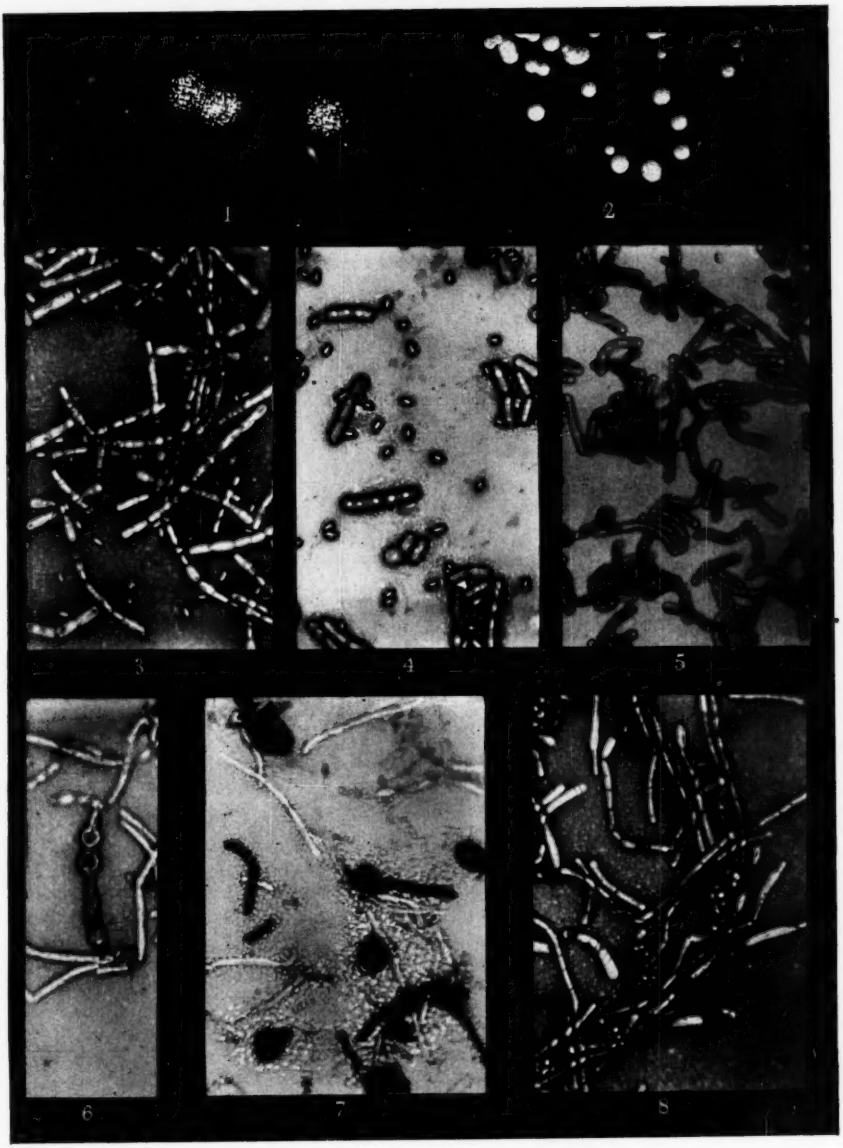
FIG. 5.—Microphotograph of film of the Hampstead variant culture grown on agar for about 30 hours. × 1250.

FIG. 6.—Globular bodies formed in a 48 hours old culture of the Hampstead variant. × 1250.

FIG. 7.—Germination of globular bodies such as seen in Fig. 6 on transfer to fresh medium. × 1250.

FIG. 8.—"Viviparous" germination of globular bodies on granules in an old culture of the Hampstead variant on transfer to a fresh agar medium. × 1250.

The preparations shown in Figs. 3–8 were double-stained with congo red and methylene blue (description of method in the press). Photographs by Mr. F. W. Welch.



White.

of the controls, which invariably died. But when the infecting dose was raised to 1/100th c.c. of the " standard " broth culture and in an experiment in which a group of animals (rabbits and guinea-pigs) which had been prepared with all zeal for the trial were given Vollum spores by the respiratory route no evidence of protection—even as witnessed by delay of death—was discernible. Moreover the survivors from the earlier experiments were not in general found to withstand a second exposure to infection. It became clear that no sound immunity could be conferred on guinea-pigs by means of such vaccines.

Looking next to Bail's well-confirmed observation that rabbits can be immunized to virulent anthrax infection by injections of sterilized oedema fluid, an effort was made to manufacture *in vitro* an effective substitute for this fluid by growing the Hampstead variant in whipped blood. The procedure adopted through the experiments discussed in detail has been as follows: 25 c.c. of pooled guinea-pig blood, obtained by cardiac puncture and shaken with beads, was transferred to a sterile 250 c.c. conical flask in which it formed a shallow layer. To the blood was added 2·5 c.c. of a sterile 10 per cent solution of Difco meat extract or of Allen & Hanbury's Beef Juice and, emulsified in a little of this blood medium, the growth of an 8 or 9 hours' old agar slope culture of the Hampstead variant. The flask, lightly plugged, was placed in a Fildes and McIntosh jar and the bulk of the air within was replaced by oxygen. The jar was then kept at 37° C. for 12–15 hours. At the end of this period the culture smeared on a slide, stained with methylene blue and examined microscopically presented some similarity to the blood of anthrax, if not to oedema fluid—capsulated bacilli scattered in a ground substance containing globules and granules of varied staining.

The blood was transferred to a constricted boiling tube, a little toluol was run in and the tube was sealed, shaken and placed in the refrigerator. At the end of 4 or 5 days, during which they had been shaken daily, the tube contents were tested for sterility, by adding one small drop of the blood to each of a series of broth tubes (30 c.c.). In most cases samples washed with broth and recovered by centrifugation were similarly tested. In no case were viable anthrax bacilli detected. The sterility tests completed, the material was stored in the cold in 4 to 5 c.c. amounts in sealed ampoules for use as required.

In the immunization of guinea-pigs 0·5 c.c. doses were given, in part subcutaneously, in part intraperitoneally, during the earlier stages of the treatment, being increased to 1 c.c. at the final injections. The course consisted of weekly doses extending over a month or 6 weeks, and the challenge was delivered a week or 10 days after the last injection. This challenge in all but one case took the form of 1/100th c.c. of the Vollum broth culture given subcutaneously in the groin; in the last experiment it consisted of 100,000 Vollum spores given intracutaneously, the inoculum being kindly supplied by Dr. G. P. Gladstone for the purpose.

The first trials were made with vaccines grown in rabbits' blood without meat extract and with guinea-pigs as test animals. Apart from a slight delay of death there was nothing in the results of these experiments to give encouragement, but two rabbits subsequently immunized by the intravenous route with like material were found to resist 1 : 100th c.c. of the virulent broth culture—a dose which killed the 2 control animals in 2 and 3 days respectively—and later withstood a much more severe challenge.

Later experiments.

With this solitary indication of success the work had to be temporarily abandoned and was not resumed till more than a year later, when the small series of experiments which forms the main substance of this note was carried out.

In these, immunization of the guinea-pig was once more attempted, this time with vaccines grown in the homologous blood and with addition of meat extract or muscle juice.

In the three experiments detailed below the challenge dose was 1/100th c.c. of Vollum broth culture given subcutaneously.

Experiment 1:

4 control animals: all died in less than 40 hours.
3 vaccinated animals: 2 died on the 6th and 10th days respectively; 1 survived and later proved solidly immune to anthrax infection (Note *a*).

Experiment 2:

3 control animals: all died in less than 40 hours.
2 vaccinated animals; 1 died on 7th day and 1 survived, withstanding several subsequent and massive injections of virulent anthrax culture (Note *a*).

Note a.—When the animals were killed, several months after the first challenge inoculation, bacteriological examination failed to discover *B. anthracis* in the spleen and other organs.

Experiment 3:

8 control animals: all died in less than 48 hours.
4 animals vaccinated with agar-grown culture: all died in less than 48 hours.
4 animals vaccinated with blood and meat extract vaccine I*: died on the 3rd, 5th, 5th and 6th days respectively.
4 animals vaccinated with blood and meat extract vaccine II*: 2 died on the 2nd and 4th days respectively; one was killed for bacteriological examination on the 15th day (Note *b*), and 1 survived till killed on the 43rd day (Note *c*).
4 animals vaccinated with blood and meat juice vaccine*: 1 died on the 6th day; 1 on the 20th day; 2 survived till killed on the 43rd day (Note *c*).

In the last experiment the challenge dose was 100,000 viable Vollum spores given intracutaneously in 0·25 c.c. of broth.

Note b.—This animal was killed on the 15th day on the presumption that specific deaths in this experiment had ceased. One colony of *B. anthracis* was obtained in a plating from the macroscopically normal spleen, and two or three colonies in a plating from the subcutaneous tissue at the site of inoculation.

Note c.—Two of the 3 survivors of Experiment 3 dropped stillborn litters; in one case 2 almost full-term foetuses on the 14th day of the experiment; in the other 5 hairless foetuses on the 33rd day. All the foetuses were massively infected with anthrax bacilli, shown later to be virulent. When the mothers were killed respectively 29 and 10 days later no anthrax bacilli could be isolated

* The animals received a final immunizing dose of 1 c.c. of a second blood and meat juice vaccine.

from either animal—not even from the uterine mucosa. The third survivor of the experiment, likewise killed and examined on the 43rd day, also appeared free of anthrax bacilli.

Experiment 4:

12 control animals: 11 died in less than 48 hours, 1 about the 50th hour.
8 animals immunized with blood and meat extract vaccine: 1 died on the 5th day, 4 on the 6th day and 3 survived.
8 animals immunized by blood and meat juice vaccine; 6 died on the 4th, 4th, 5th, 6th, 9th and 10th days respectively and 2 survived.

The summed results of the four experiments are:

	Controls.	Vaccinated animals.
Number of animals employed . . .	33 (37)*	33
Average time to death of animals dying .	2 days	6·5
Survivors	0	10 (11)†

* Including 4 animals immunized with agar grown vaccine.
† Including 1 animal killed for examination when in good health on the 15th day of the test.

It must be stated that in another experiment—justifiably, I think, excluded from the above analysis—8 animals which had been immunized with a vaccine prepared on the usual lines, but in which citrated blood was substituted for whipped blood, all died without distinction from the controls. It was noted that little multiplication of the bacilli in the medium had occurred, and that the capsulation of the rods was poorly and irregularly developed.

DISCUSSION.

The experiments recorded are small in number and extent, and the success of the immunizing process but partial. In the ordinary way the matter would not be presented for publication at this stage. Nevertheless the results seem significant, and it is felt that the induction of a tangible measure of immunity to anthrax in even a percentage of animals—about one-third of those treated—with a very definite stay of death in the remainder, is sufficiently remarkable to be worth recording in conjunction with the publication of Dr. Gladstone's results (Gladstone, 1946). Moreover, the observations must now again be discontinued for some months.

There is clearly far to go before a group of guinea-pigs can be immunized with a non-living vaccine with a confident expectation that all or the majority will thereafter be protected soundly against an infecting dose of anthrax bacilli or spores, such as I have employed in making the challenge; but that the guinea-pig—or certain guinea-pigs—can be protected against such infection without the intervention of a living agent is now equally clear.

It is possible that the high grade of immunity seen in two rabbits and the surviving guinea-pigs from the experiments listed as 1 and 2 which all withstood with equanimity a second and relatively massive inoculum of virulent culture, and in the two survivors of Experiment 3, which harboured with impunity anthrax-infected foetuses, was partially engendered by the infection subdued at the first and more moderate challenge. Whether such complete insusceptibility, in which

the size of the test inoculum is of little account, can be attained directly by the type of vaccination I have practised is a matter for later trial.

The anthrax infected litters born of healthy mothers have a bizarre interest. It may be wondered whether the foetuses were infected through the uterine wall or placenta or by ascent of the genital tract. Both the females concerned were caged with males which died of the disease. Dr. Gladstone has suggested to me that this foetal susceptibility, coupled with maternal immunity, may arise from the cellular as opposed to the humoral nature of the latter.

To discuss the factors possibly concerned in the immunity observed would be beside the mark till more extensive and incisive study has been made. It seems likely that the soluble factor active in Dr. Gladstone's experiments is involved, and this may well be the main agent concerned. To the defensive reaction which it excites may be merely added such antibacterial response (anti-bacillary and anticapsular) as might be stimulated by an ordinary agar-grown vaccine—a resistance puny in itself, but serving perhaps here and there to tip the scales of life and death.

These observations on anthrax in particular beg the question as to whether in the wider field of bacterial prophylaxis there may not be other instances in which a vaccine grown under conditions approximating to those of parasitism may have immunizing virtues lacking in a broth or agar culture. The situation in regard to bacterial vaccines is not so satisfactory as to render investigation of the possibility unworthy of the essay.

SUMMARY.

1. The features of a variant of *B. anthracis* (strain Vollum) which fails to form thermo-resistant endospores are described.

2. Guinea-pigs intensively immunized with agar grown cultures of this variant sterilized by alcohol and heat were sometimes able to resist infection by doses in the order of 1/5000 c.c. of virulent broth culture which killed all controls, but such survivors were not as a rule immune to a second and larger inoculum, and when the challenge dose was increased to the order of 1/100th c.c. of virulent broth culture the immunizing treatment was utterly ineffective.

3. Two rabbits and 10 (or 11) of 33 guinea-pigs (comprising 4 experiments) which were immunized over a month or 6 weeks with toluol killed cultures of the variant grown in homologous blood with addition of meat extract or meat juice, and in an atmosphere of oxygen survived challenge with 1/100 c.c. of virulent broth culture or 100,000 viable spores, which dose killed all controls, inoculated in comparable number, within two days. The average time to death of the immunized animals which succumbed was prolonged to 6·5 days. Those surviving animals which were tested were found solidly immune to subsequent heavy inoculation with anthrax bacilli and there was evidence of the similar immunity of others.

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THE COMPONENTS OF THE SERUM PROTEINS IN INFECTIVE HEPATITIS AND IN HOMOLOGOUS SERUM JAUNDICE (AN ELECTROPHORETIC STUDY).

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Over the last twenty years a steadily increasing volume of literature has stressed the importance of the liver in protein metabolism (Bollman and Mann, 1936; Madden and Whipple, 1940; Post and Patek, 1943). In an analysis of a series of fatal cases of hepatitis, Lucké (1944) found that the constant predominating lesions were confined to the liver. Similar lesions had been observed previously in material obtained from the livers of living patients (Roholm and Iversen, 1939; Dible, McMichael and Sherlock, 1943). In such cases, until recently, protein analysis has been confined to an estimation of the total proteins, supplemented by a study of the albumin globulin ratio using the method of Howe. In 1943 the electrophoretic method of Tiselius was applied in the analysis of isolated samples of plasma from five cases of "catarrhal" jaundice by Gray and Barron. These analyses were carried out at a pH of 7·8, and therefore were not calculated to give the optimal resolution of the α globulins. In order to form a base line for further work on protein metabolism in infective hepatitis, the following serial electrophoretic patterns of the serum proteins have been recorded in a thoroughly documented group of patients suffering from uncomplicated infective hepatitis, to establish the character and extent of alteration of that pattern and their correlations with other findings in this disease.

CLINICAL MATERIAL.

The material in the study consists of a group of eight males and three females drawn from the wards of the Rockefeller Institute for Medical Research, New York, and the Peter Bent Brigham Hospital, Boston. Their ages varied from 16·5 to 44 years, and they all had the typical initial symptoms of nausea and anorexia, with some fever. They all developed icterus and passed bile-stained urine. Their course was considered to be that of uncomplicated infective hepatitis. It should be noted that four of the males had received convalescent serum for mild attacks of scarlet fever eight weeks previous to the onset of their icterus. It is almost certain that the infecting agent had been transmitted to the four by serum inoculation. No difference was noted in these patients from other members of the group either in the course and character of the disease or in the character of the electrophoretic pattern.

MATERIAL AND METHODS.

All samples of serum were stored in liquid state at 2° C. prior to dialysis. Dialysis was carried out for 72 hours against sodium diethyl barbiturate buffer of 0·1 ionic strength and pH 8·6, two changes of buffer being made. Analyses were performed in the apparatus of Tiselius (1930). The long cell of Tiselius, as described by Longsworth (1942), being especially suitable for analytical procedures, was used. The concentration of protein in the solutions for electrophoresis was adjusted to between 1·75 per cent and 2·1 per cent of protein immediately prior to analysis, in order to minimize the apparent alterations in the percentage of individual components produced by variations in total protein concentration (Perlmann and Kaufmann, 1945). As far as possible the serial runs on individual patients were kept within even closer limits. By this means apparent variations in albumin percentages ascribable to small differences in salt or protein concentrations should fall within a range of 3 per cent. Armstrong, Budka and Morrison (in press) has pointed out a more serious discrepancy that may arise from an attempt to calculate the absolute values of individual components from a total protein based on Kjeldahl estimations, using a nitrogen factor of 6·25, which arises in part from the variable amounts of bound lipid in the different fractions. In the table of analyses the components as presented are susceptible of correction in the light of these observations.

RESULTS.

The results are shown in Table I.

In this group of cases of infective hepatitis the depression of the albumin fraction was associated with an increase in gamma globulin, the extent of the depression in albumin being mirrored approximately by the gamma globulin increase. Two of the patients in whom there was the most marked drop in albumin required prolonged hospitalization before recovery, but their recovery was without incident and they have shown no tendency to relapse. They were the two oldest members of the group. Two patients who ran a protracted and relapsing course had no significant alteration in their albumin levels as compared with others in the group who had mild attacks with rapid complete recovery. In general, the albumin fraction showed its maximum depression in the first ten days from the onset of symptoms, recovering steadily thereafter. It is suggested that it reflects the extent of temporary parenchymatous damage. In the group examined the degree and duration of anorexia did not correlate with the alteration in serum albumin. Those with most marked alteration had no greater anorexia than the others.

The γ globulin reaches its peak as the albumin falls, dropping again as the albumin recovers, but not returning to normal in every instance. Bjørnboe (1946) has attempted to demonstrate that a linear relationship exists between the serum albumin and serum globulin in cases of hepatitis without ascites or oedema, according to the formula $2{\cdot}52A + G = 13{\cdot}1$. While some general relationship may well exist, as yet it is by no means proved, and it was not possible in this series to demonstrate any linear relationship. The rise in γ globulin may be attributable to three mechanisms at work: (*a*) a compensatory rise in an attempt to maintain osmotic relationships, or (*b*) the possible formation of antibodies (Eaton, Murphy and Hanford, 1944), or (*c*) some alteration in the relative

TABLE I.

Patient.	Day from onset of symptoms.	Total protein (g./100 ml.).	A/G salted out.	A/G electroph.	Alb./T.	α_1/T.	α_2/T.	β/T.	$\alpha_2+\beta$/T.	γ/T.	B.S.P.[2]	Bili. (mg. per cent).	Thymol turbidity.
T. J—	9	—	—	0·98	0·47	0·053	0·059	0·18	0·24	0·18	38·5	9·6	21·5
	21	7·3	—	0·98	0·47	0·049	0·047	0·22	0·267	0·16	12·5	2·2	12·3
	29	6·2	—	1·25	0·55	0·05	0·077	0·14	0·217	0·14	5·0	1·5	7·8
W. M—*	6	—	—	1·05	0·51	0·047	0·11	0·14	0·25	0·17	38·5	10·8	19·5
	16	7·5	1·64	1·05	0·51	0·05	0·095	0·18	0·28	0·16	13·0	2·4	11·0
	23	7·3	1·44	1·05	0·51	0·045	0·15	0·10	0·25	0·17	6·5	1·6	8·4
	34	7·36	1·45	0·99	0·48	0·03	0·08	0·16	0·24	0·18	3·5	1·3	6·8
	44	—	—	1·1	0·52	0·03	0·12	0·14	0·26	0·17	1·0	0·65	5·8
B. L—*	9	—	—	0·7	0·41	0·032	0·10	0·16	0·26	0·26	27·0	6·6	34·0
	21	—	—	0·99	0·49	0·043	0·11	0·15	0·26	0·18	9·5	1·9	18·8
	29	—	—	1·2	0·54	0·041	0·114	0·13	0·244	0·15	3·0	1·0	14·0
	43	—	—	1·3	0·56	0·025	0·11	0·13	0·24	0·155	0·5	0·75	6·8
	50	—	—	1·5	0·59	—	0·147	0·09	0·24	0·15	3·0	0·60	7·8
J. P—	6	6·27	1·33	1·25	0·55	0·04	0·067	0·15	0·22	0·17	48·0	12·0	—
	13	6·42	1·26	1·1	0·52	0·035	0·070	0·20	0·27	0·15	27·0	5·6	8·4
	20	6·83	1·85	1·25	0·55	0·045	0·070	0·20	0·27	0·12	8·0	2·2	4·6
	33	6·93	2·1	1·7	0·63	—	0·088	0·13	0·22	0·12	7.0	1··8	4·3
E. D—*	6	6·54	1·87	1·2	0·53	0·045	0·045	0·14	0·21	0·20	34·5	2·2	13·0
	22	7·45	2·05	1·6	0·61	0·033	0·09	0·14	0·23	0·12	11·0	0·5	6·0
	29	7·83	2·19	1·4	0·58	0·026	0·15	0·096	0·24	0·12	7·0	0·2	6·8
W—	9	—	—	0·99	0·49	0·04	0·20	0·10	0·30	0·15	43.0	14·2	11·6
	17	—	—	1·15	0·53	0·02	0·13	0·15	0·28	0·12	41.0	11·2	12·3
	23	—	—	1·3	0·56	0·046	0·11	0·14	0·25	0·12	30.0	4·6	9·5
	30	7·18	2·0	1·7	0·63	0·022	0·07	0·14	0·21	0·12	16.0	2·0	10·5
	64	—	—	—	0·62	0·04	0·08	0·13	0·21	0·10	8.0	0·35	10·0
R. W—	10	—	—	0·99	0·49	0·03	0·05	0·135	0·185	0·25	45.0	10·2	20·6
	16	—	—	0·97	0·44	0·04	0·104	0·17	0·274	0·23	15·5	3·6	32·0
	23	7·7	1·1	0·98	0·46	0·03	0·12	0·15	0·27	0·23	8·5	1·95	21·5
	30	7·18	1·77	1·25	0·55	0·02	0·06	0·145	0·205	0·20	6·5	1·3	18·0
	37	—	—	1·4	0·58	0·03	0·07	0·12	0·19	0·19	—	—	11·9
	50	6·72	2·0	1·5	0·56	0·04	0·07	0·11	0·18	0·21	13·5	—	12·0
	57	8·05	—	1·5	0·56	0·03	0·04	0·12	0·16	0·24	19·5	0·9	17·0
H. B—*	8	6·69	1·33	1·2	0·53	0·05	0·08	0·17	0·25	0·17	42.0	10·8	21·5
	15	6·87	1·20	1·0	0·50	0·04	0·17	0·16	0·33	0·20	28·5	5·4	23·0
	22	6·7	1·60	1·5	0·56	0·03	0·11	0·16	0·27	0·13	26·0	2·9	13·8
	31	—	—	1·5	0·56	0·04	0·10	0·13	0·23	0·12	7·5	1·2	9·0
G—	15	—	—	0·9	0·38	0·049	0·091	0·15	0·24	0·26	41.0	3·17	18.0
	30	—	—	0·98	0·47	0·053	0·126	0·13	0·25	0·18	45.0	0·99	16.0
	48	—	—	1·6	0·61	0·042	0·06	0·11	0·17	0·11	40.0	0·61	—
K—	16	—	—	0·95	0·40	0·03	0·07	0·21	0·28	0·24	55.0	8·41	32.0
	23	—	—	0·97	0·45	0·045	0·08	0·185	0·26	0·195	—	—	20.0
	29	—	—	0·98	0·48	0·03	0·095	0·17	0·26	0·175	32·5	24·51	19·0
	41	—	—	1·0	0·50	0·03	0·085	0·18	0·26	0·177	11·0	—	16.0
	58	—	—	1·1	0·53	0·035	0·08	0·13	0·21	0·21	13·5	—	—
B—	9	—	—	0·95	0·40	0·03	0·06	0·20	0·26	0·25	33.0	5·46	36.0
	16	—	—	0·96	0·43	0·03	0·07	0·18	0·25	0·24	16.0	—	—
	23	—	—	1·0	0·50	0·03	0·095	0·17	0·265	0·22	22·5	—	40.0
	39	—	—	1·5	0·59	0·05	0·09	0·15	0·24	0·13	—	—	25.0
Normal values—serum					0·58-0·62	0·035-0·05	0·06-0·09	0·11-0·14	0·17-0·23	0·10-0·14			

The first eight patients are from the series investigated at the Rockefeller Institute for Medical Research.

* These patients had received convalescent serum approximately 8 weeks before the onset of symptoms of infectious hepatitis.

[1] Albumin/globulin ratios salted out by the technique of Howe.

[2] B.S.P. represent clearance at 45 minutes, 5 mg. per kg. body weight of bromsulphalein being given, with exception of cases G—, K— and B—, in whom clearance was taken at 30 minutes on a 5-mg. dose per kilogram.

rates of production and utilization of the albumin and globulin fractions of the serum proteins in infective hepatitis. There seems no reason to suppose that the three would in any way be connected, and it is not to be expected therefore that, since antibody production will vary from one patient to another, a linear relationship would hold in all cases.

The alpha$_2$ and beta globulins followed no such constant pattern; one or both were elevated at some stage of the disease, usually between the 14th and 30th day from the date of onset of the first symptoms. The fluctuations in the level of alpha$_2$ and beta in individual patients varied considerably, and it was noted

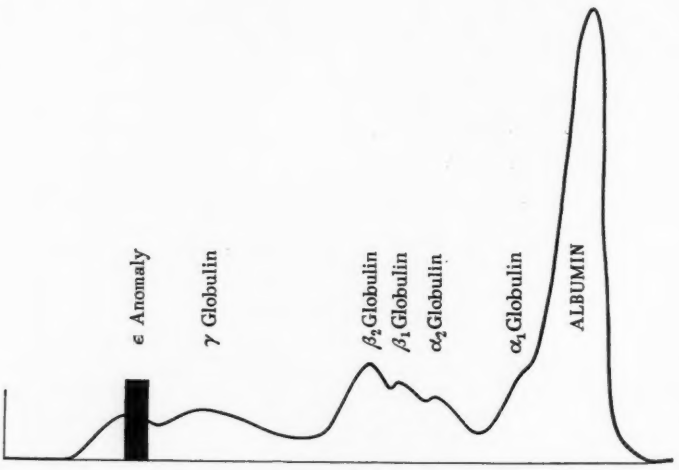

FIG. 1.

	Alb.	α_1.	α_2.	β_1.	β_2.	γ.
Concentration (ratio of component to total area less anomaly)	0·515	0·036	0·07	0·07	0·14	0·16
Mobility (cm.^{2} volt. sec.)	5·8	4·8	4·07	3·5	3·0	1·1

that in the two cases which ran prolonged courses with tendency to relapse this fluctuation was most pronounced, 0·114 to 0·09 compared with 0·02 to 0·05 in the milder cases. During the analysis it was noted that in certain sera there was a tendency for the beta globulin complex to resolve into a fast and a slow component. Two examples are shown (Fig. 1). From calculations of the mobilities there is no retardation of the alpha$_2$ globulin, the second peak probably being due to a fast moving component of the beta complex. This phenomenon could not be correlated convincingly with any clinical findings. That it was not a function of storage is shown by the fact that sera from comparable stages of the disease stored under the same conditions and for the same length of time showed no such breakdown, and that it tended to occur in the serial sera of the individual patients irrespective of the length of time that the given sera had been stored. It is not without interest that it is the beta globulin complex that contains a significant percentage of the lipid carrying proteins of the blood stream

(Blix, Tiselius, and Svensson, 1941), and that the ratio of free and bound cholesterol in infective hepatitis deviates significantly from normal (Hoagland and Schank, 1946). There were no significant changes in the alpha$_1$ globulin component. At the time the samples were taken all the patients were afebrile. In some sera alpha$_1$ globulin did not separate cleanly from albumin.

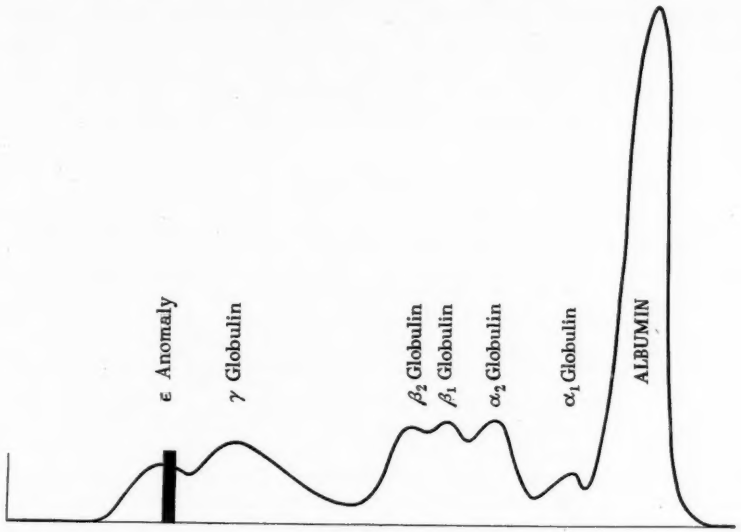

FIG. 2.

	Alb.	α_1.	α_2.	β_1.	β_2.	γ.
Concentration (ratio of component to total area less anomaly)	0·51	0·05	0·095	0·09	0·09	0·16
Mobility (cm.^{2} volt. sec.)	5·98	5·0	4·05	3·1	2·85	1·5/0·85

DISCUSSION.

The results obtained in the serial analyses are broadly in line with five isolated analyses published by Gray and Barron in 1943. It must be stressed at once that they are in no way peculiar to infective hepatitis. Similar patterns have been obtained in infective mononucleosis (Cohn and Ledimann, 1946), in lupus erythematosus and in other conditions (unpublished data, Dr. S. H. Armstrong, Physical Chemistry Laboratory, Harvard Medical School). They are not, therefore, an absolute diagnostic aid. Their value lies in forming a base line for further studies of the administration of protein fractions in the treatment of the condition and in assessing the effect of those fractions, if any, on the plasma proteins.

SUMMARY.

(1) In eleven patients serial electrophoretic examination of the serum proteins was made. Depression of the albumin component with a corresponding rise of gamma globulin was noted.

(2) There was disturbance of the alpha$_2$ and beta globulin, notably between the 14th and 30th days. It was greatest in two patients who ran a relapsing course.

(3) A breakdown in the beta complex was noted in certain sera, but it appeared to have no diagnostic significance.

My thanks are due to Prof. E. J. Cohn and the Department of Physical Chemistry, Harvard Medical School, for placing their facilities at my disposal, to the Rockefeller Institute for Medical Research, and particularly Dr. C. L. Hoagland for material and data on individual cases, and to the staff of the Peter Bent Brigham for allowing me access to their cases.

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THE PREPARATION AND USE OF COLLOIDAL GOLD SOLS AS DIAGNOSTIC AGENTS.

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COLLOIDAL gold sols have been used extensively in testing cerebrospinal fluid since the work of Lange (1912, 1945), and have also been employed as a liver function test on serum by Gray (1940) and Maclagan (1944*a*). Experiments with electrophoretically separated protein fractions indicate that positive results with the test depend, both in serum and in cerebrospinal fluid, upon a relative increase in the gamma globulin concentration, albumin having an inhibitory effect (Gray, 1942; Kabat, Moore and Landow, 1942). There is also evidence that different albumin preparations vary in their inhibitory power (Kabat, Hanger, Moore and Landow, 1943). More recently Maclagan and Bunn (1946) have shown that α and β globulins inhibit the reaction even more powerfully than albumin, so that alterations in all four fractions may be of importance. Nevertheless it appears probable that variations in the gamma globulin content is the most important single factor to be concerned, and it is noteworthy that absolute increases in this fraction are known to occur in those diseases in which the test has been particularly applied. Thus the gamma globulin concentration is usually raised in the serum in parenchymatous liver diseases (Gray and Barron, 1942) and in the cerebrospinal fluid in neurosyphilis and in disseminated sclerosis (Kabat *et al.*, 1942).

The routine application of the tests has, however, been somewhat restricted by technical difficulties (Mateer, Baltz, Marion and Hollands, 1942). Thus the sensitivity of a gold sol preparation depends upon the particle size of the sol and upon the pH and ionic strength of the medium (Glazoe and Sorum, 1940; Lange and Harris, 1944; Maclagan, 1944*a*), and these variables have rarely been accurately controlled. If, however, the pH and ionic strength are fixed by a suitable buffer solution, as in the serum colloidal gold reaction recently described (Maclagan, 1944*a*), it is essential to use a sol of reproducible particle size. For the last four years the writer has used the oxalate method of Patterson (1931), and this appeared to be perfectly satisfactory until a change of laboratory led to the use of a different distilled water supply, when repeated failures were encountered. After many experiments the conclusion was finally reached that Patterson's (1931) method as described could not be made to work with water distilled from an all-glass still. The early darkening of the mixture which is an essential part of the method appeared to depend upon traces of catalyst derived from the cork connections, and while the effect could be imitated by substances such as gallic acid (0·1 mg. per litre) the reaction was difficult to standardize, and it appeared

desirable to present an alternative preparation. The citrate methods of Lange (1939), Bartholomew and Gent (1940) were next tried, but of these the former gave a sol of poor keeping qualities which was very blue in colour, and the latter one which was much less sensitive than the oxalate sol when tested at the same pH. Finally a slight modification of the citrate method was developed which gives a gold sol nearly as sensitive as the oxalate sol, and appears to be independent of the source of distilled water and of the rate or order of the addition of reagents. The required sensitivity can easily be achieved by using a slightly more acid buffer solution than the one previously described in the final test.

The principal modification introduced is an alteration in the amount of sodium citrate used. It was found after numerous trials that smaller amounts of citrate than those recommended here gave more sensitivity, but that the sol became less sensitive on keeping, as noted by Lange (1945); larger amounts on the other hand produced a less sensitive sol which became more sensitive on keeping. The present sol has maximum stability and is sufficiently sensitive for use as described.

Preparation of Gold Sol.

Solutions.

Gold chloride 1 *per cent.* 1 g. sodium gold chloride made up to 100 ml. with distilled water.

Sodium citrate 1·6 *per cent.* 1·6 g. sodium citrate analytical grade made up to 100 ml. with distilled water.

Double distilled water. Ordinary distilled water redistilled from an all glass still. Water from a tin-lined still has also been used with identical results, but may be less certainly reproducible in other laboratories.

Method.

The procedure is described for 500 ml. lots, but proportionately larger or smaller amounts may be used. Place 500 ml. of double distilled water in a suitable flat-bottomed flask and bring to the boil. When boiling briskly add 5 ml. of 1 per cent gold chloride and 5 ml. of 1·6 per cent sodium citrate solution. After allowing a few seconds for mixing, reduce the heating so that the mixture simmers gently, place a funnel in the mouth of the flask to minimize evaporation, and continue simmering until the full sequence of colour changes is complete (about 5 minutes). Then cover the mouth of the flask and allow to cool. The colour changes referred to commence with a dusky blue, changing to dark purple, and finally lightening slowly to a clear red. The final sol is a clear red and shows no obvious Tyndal effect or "sheen" in ordinary daylight. The pH is about 5·9 (glass electrode).

Storage.

The sol is stored in the flask used for preparation, which is covered with an inverted beaker and kept in a dark cupboard. It must not be pipetted direct from the flask, and unused portions must not be returned to the main bulk. Under these conditions it will keep several months without change.

Serum Colloidal Gold Reaction.

The procedure is similar to that previously described (Maclagan, 1944*a*), except that the pH of the buffer is reduced from 7·8 to 7·7.

Barbitone buffer. pH 7·7, ionic strength 0·015.

Barbitone (diethyl barbituric acid)	0·55 g.
Sodium barbitone (Na salt of above)	0·31 g.
Phenol	0·2 g.

Add 100 ml. of distilled water and dissolve by gentle heating.

Test.

Measure 0·05 ml. of serum from a 0·2 ml. graduated pipette with a waxed tip into a clean 4 by $\frac{5}{8}$-in. test-tube, add 0·5 ml. of buffer and 2·5 ml. of gold sol. Shake and allow to stand overnight. Complete precipitation of gold sol is recorded as 5+, slight precipitation only visible on shaking the tube gently as 1+, and intermediate grades of precipitation as 2+, 3+, or 4+, depending chiefly on the colour of the supernatant fluid. Normal serum gives no precipitation, recorded as 0 or negative.

Comparison with Oxalate Method at pH 7·8.

Duplicate tests on a variety of normal and abnormal sera have given almost identical results with the two methods, as shown in the following table :

Serum number .	1	2	3	4	5	6	7	8	9	10
Oxalate sol pH 7·8 .	5	5	5	4	3	2	2	0	0	0
Citrate sol pH 7·7 .	5	5	4	4	4	2	2	0	0	0

The interpretation of results with the new method is therefore governed by the same principles as those already given (Maclagan, 1944*a*, 1944*b*; Carter and Maclagan, 1946).

Buffers for Use with Cerebrospinal Fluid.

Although the citrate sol can be used without buffers by the usual process of standardizing against known positive and negative fluids with addition of small amounts of alkali,* there are obvious advantages in employing a suitable buffer. These are that the reaction is then unaffected by traces of acid or alkali, and that known fluids are not needed for standardization of the sol. Lange (1939) proposed a buffer of pH 7·4 for use with his special citrate sol, but the method does not appear to have been generally followed.

In order to find the most suitable buffer for use with the oxalate sol, a typical normal cerebrospinal fluid was put up by the usual Lange (1912) technique, using phosphate buffer solutions in place of 0·4 per cent NaCl, with the results shown in Table I.

* The citrate sol as prepared by the writer requires the addition of about 0·2 ml. of 0·02N NaOH per 15 ml. of sol if it is to be used with 0·4 per cent NaCl as diluent, but this volume might show some variation with different sources of distilled water.

TABLE I.—*Effect of Varying pH and Ionic Strength with Normal C.S.F. Oxalate Sol.*

Ionic strength.	pH of phosphate buffer. 6·3.	6·8.	7·3.
0·067	000000	000000	000000
0·1	000010	000000	000000
0·15	000120	000100	000000
0·2	013120	022000	000000
0·3	—	123330	010000
0·4	—	134321	020000

It would appear from Table I that with cerebrospinal fluid a similar relation between sensitivity, pH and ionic strength exists as previously demonstrated for the serum colloidal gold reaction (Maclagan, 1944*a*). Lowering pH or raising ionic strength both have the effect of increasing sensitivity, and the three buffers underlined in Table I (6·3/0·067, 6·8/0·1, 7·3/0·2) just give negative results with normal fluids. These three were therefore chosen for further trial with pathological fluids, with the result given in Table II.

TABLE II.—*Trial of Three Possible Buffers with Abnormal Fluids. Oxalate Sol.*

	$\frac{6\cdot3}{0\cdot067}$	$\frac{6\cdot8}{0\cdot1}$	$\frac{7\cdot3}{0\cdot2}$	Original Lange.
Diagnosis.				
Normal	000000	000000	000000	000000
General paresis	555442	555443	333554	555443
Tuberculous meningitis	333221	111233	001244	255533
Meningococcal meningitis	000011	000011	000123	000012
Spinal block	441331	111133	001344	221233
Diluted serum*	551021	000020	000033	055423

* The dilution factor from tube to tube in this experiment was $\frac{2}{3}$ in place of the usual $\frac{1}{2}$.

It will be seen from Table II that of these three the buffer of pH 6·8, ionic strength 0·1, appears to be the best. While paretic fluids give equally positive results at pH 6·8/0·1 or pH 6·3/0·067, the latter tended to give more false positives with meningitic fluids and with diluted serum, and the pH 7·3/0·2 buffer was undesirable because of zone effects.

The 6·8 buffer was therefore adopted for use with the oxalate (Patterson) sol and is made up as follows:

Buffer for oxalate sol pH 6·8, *ionic strength* 0·1.

KH_2PO_4 anhydrous 0·340 g.
Na_2HPO_4 anhydrous 0·355 g.
Distilled water to 100 ml.

This buffer keeps well for several weeks, but should be renewed if moulds grow in it. It is convenient to prepare the buffer as a solution ten times the above strength, and dilute for use as required.

The citrate sol as described here is rather less sensitive, and requires the more acid buffer given below.

Buffer for citrate sol, pH 6·6, *ionic strength* 0·067.

KH_2PO_4 anhydrous 0·314 g.
Na_2HPO_4 anhydrous 0·207 g.
Distilled water to 100 ml.
Renew if moulds grow.

Method.

Measure 0·9, 0·5, 0·5, 0·5, 0·5 and 0·5 ml. of buffer into 6 clean test-tubes 4 by $\frac{5}{8}$ in. Add 0·1 ml. of cerebrospinal fluid to first test-tube, mix and transfer 0·5 ml. to second tube, and so on until the last 0·5 ml. is discarded. Then add 2·5 ml. of gold sol to each tube, shake and allow to stand overnight. Record results as in ordinary Lange test in which 5 = complete precipitation of gold sol. Precipitation rarely occurs after the 6th tube so that the conventional 10 tubes are unnecessary.

Comparison of the Two Sols.

Duplicate experiments with a number of abnormal fluids have shown no appreciable difference between the results when the oxalate sol was used with the 6·8 buffer, as compared with those obtained when the citrate sol was used with the 6·6 buffer.

Precipitation of Gold Sols by Pure Gamma Globulin.

It is evident that the sensitivity of the sols must be enormously greater at pH 6·6 or 6·8, as used for cerebrospinal fluid, than at pH 7·7 or 7·8, as used for serum, corresponding to the different amounts of protein in the two fluids. This has been demonstrated in some experiments reported elsewhere (Maclagan and Bunn (1946), in which increasing dilutions of the electrophoretically pure gamma globulin prepared from normal serum were put up with gold sol by the methods described above. In this way it was found that 75 μg. of globulin were required for complete precipitation of 2·5 ml. of the citrate sol at pH 7·7, whereas 0·2 μg. were sufficient at pH 6·6. This increase in sensitivity with falling pH is probably due to decrease in negative electric charge on the gold particle together with an increase in the number of positive charges on the protein molecule. It is important to note that the net charge in the case of gamma globulin would still be negative at pH 6·6, since its isoelectric point is about pH 6·0. This protein is the only one known to absorb against an electric field in this way (Elkes, Frazer, Schulman and Stewart, 1945).

Results with Pathological Fluids.

Tables III and IV show the results obtained by the buffer method with some typical pathological fluids.

It will be seen from Tables III and IV that positive curves may be roughly classified as first zone or end zone in character, according to whether maximal precipitation occurs in the first 3 or last 3 tubes. End zone precipitation is usually feeble, and rarely exceeds 3+ in intensity. In addition to the results shown in

the tables a large number of fluids of varying protein content have given completely negative results. So far no fluid with normal protein content (less than 40 mg. per cent) has given a positive result except those given in Table III.

The test is recognized as being of most value as an indication of neurosyphilis or disseminated sclerosis, and it will be seen from Table III that a high proportion

TABLE III.—*Buffered Cerebrospinal Fluid Gold Curves in 34 Cases of Neurosyphilis and Disseminated Sclerosis.*

Case No.	Diagnosis.	Cells per cu.mm. R.B.C.	Cells per cu.mm. W.B.C.	Protein (mg. %.)	Wassermann reaction.	Gold curve.
1.	General paresis	0	24	80	++++	555341
2.	,, ,,	0	..	..	++++	555410
3.	,, ,,	0	1	120	++++	555412
4.	Taboparesis	0	10	250	++++	554310
5.	,,	0	10	65	++++	555310
6.	,,	0	280	55	+++0	554333
7.	,,	0	14	35	anticomplementary	554321
8.	General paresis post malaria	0	28	30	+000	412000
9.	Tabes	0	1	30	++00	001210
10.	,,	0	0	10	0000	002242
11.	,,	0	22	45	++00	000100
12.	,,	0	40	30	++++	443310
13.	,,	0	1	15	..	333111
14.	,,	0	16	25	++00	000222
15.	Meningovascular syphilis	0	30	800	++++	555442
16.	Ditto	0	120	100	++++	442244
17.	,,	0	62	60	++++	443311
18.	,,	0	..	30	0000	433100
19.	,,	0	30	70	++++	333332
20.	Disseminated sclerosis	0	0	60	0000	555310
21.	,, ,,	0	5	20	0000	553210
22.	,, ,,	0	1	25	0000	533311
23.	,, ,,	0	3	15	0000	531000
24.	,, ,,	0	1	30	0000	543100
25.	,, ,,	0	2	25	0000	542100
26.	,, ,,	0	0	70	0000	433321
27.	,, ,,	0	0	30	0000	431222
28.	,, ,,	0	2	30	0000	422000
29.	,, ,,	0	1	15	0000	333321
30.	,, ,,	0	1	15	0000	222221
31.	,, ,,	0	0	30	0000	211000
32.	,, ,,	0	0	36	0000	111110
33.	,, ,,	0	0	35	0000	000111
34.	,, ,,	0	0	30	0000	000000

TABLE IV.—*Cerebrospinal Fluid in* 16 *Cases with Raised Protein Content, Neurosyphilis and Disseminated Sclerosis Excluded.*

Case No.	Diagnosis.	Cells per cu.mm. R.B.C.	Cells per cu.mm. W.B.C.	Protein (mg. %.)	Buffer method.	Lange method.
1.	Pyogenic meningitis .	0	24,000	480	111100	..
2.	,, ,, .	0	5,000	350	000011	00001, 22100
3.	,, ,, .	..	..	..	000000	22210, 22210
4.	,, ,, .	0	2,340	80	200000	430000
5.	,, ,, .	..	470	60	000210	..
6.	Tubercular meningitis .	0	210	300	111233	25553, 33210
7.	Spinal block . .	0	0	340	000000	..
8.	,, ,, . .	0	0	125	000000	..
9.	,, ,, . .	0	8	160	111133	..
10.	,, ,, . .	0	0	120	000012	20002, 20000
11.	,, ,, . .	0	1	400	000000	..
12.	Brain tumour . .	0	0	300	133112	45543, 20000
13.	Epilepsy—fluid contaminated with blood	2,300	..	40	000000	22330, 00000
14.	Ditto . .	67,000	..	120	111000	55553, 31100
15.	,, . .	610	..	30	000000	122000
16.	Cerebral haemorrhage .	21,000	..	360	000000	02210, 11100

of positive results were recorded in these conditions, about 79 per cent of cases giving first zone curves. It is of some importance to consider the results in relation to the total protein content of the fluid, as pointed out for the ordinary Lange technique by Merritt and Fremont-Smith (1937). Thus while a first zone curve appears to be of diagnostic significance at all protein levels (Table III), an end zone curve may occur in any condition which raises the protein content of the fluid, such as purulent meningitis, and spinal block (Table IV). End zone curves with normal protein content are, however, suggestive of neurosyphilis (Table III, Cases 9, 10, 14).

If the results are interpreted in this way, the test appears to be reasonably free from interference by contamination of the fluid with blood proteins or purulent exudate, and " false " positive results are unusual. The advantage of the buffer technique over the original Lange method in this respect is further illustrated in the last column of Table IV, which shows 10 cases giving " false " positive results by the original technique in which the findings were negative or nearly negative with the buffer. These were cases of purulent meningitis, cerebral tumour, and accidental contamination of the fluid with blood, in which no useful significance attached to the positive findings.

DISCUSSION.

It is hoped that the technical modifications proposed above will remove most of the uncertainties which have formerly been associated with the use of gold sols as diagnostic agents (Mateer *et al.*, 1942). The combination of a buffer solution with a method of preparation of the sol which appears to yield a constant

particle size have made the test independent of the need of known fluids or sera, and of the presence of traces of acid or alkali. While in the case of serum the cephalin cholesterol test (Hanger, 1939) and the thymol turbidity test (Maclagan, 1944*c*) have become serious rivals to colloidal gold, there are still advantages in using gold in conditions such as rheumatoid arthritis, malaria, glandular fever, and infective endocarditis, where the proportion of positive results is higher with the gold than with other tests (Carter and Maclagan, 1946). In the case of cerebrospinal fluid the extreme sensitivity of the gold reaction in the pH region of 6·6 to 6·8 gives it a unique value, making it the only flocculation test effective with small volumes (0·1 ml.) of fluid.

The historical background of the cerebrospinal fluid test has recently been reviewed by Lange (1945). The theoretical and practical aspects of pH measurement and control by buffer solutions had not been worked out in 1912 when Lange's original description appeared, and this has led to the perpetuation of a procedure which is most undesirable from a chemical aspect. The usual practice of diluting the fluid with 0·4 per cent saline means that the results are largely dependent upon the pH of the sol as prepared, and this is usually quite uncontrolled and liable to large fluctuations from trivial causes. Moreover the first tubes in the series are always more alkaline than the last tubes owing to the alkalinity of the cerebrospinal fluid, and this variation tends to produce zone effects since the sensitivity of the reagent is reduced in the earlier tubes.

The advantages of using a buffer in cerebrospinal fluid tests are therefore obvious ; in addition the buffer technique has the effect of minimizing the traditional differences in types of reaction, the majority of positive curves being " paretic " or first zone in type. There are two reasons why this may be advantageous rather than the reverse. Firstly from the chemical standpoint, the precipitation of the gold sols appears to depend principally upon a relative or absolute increase in the gamma globulin content of the fluid, as noted above. If therefore one accepts the test simply as an indication of this property, the significance of qualitative differences is obscure. Secondly from the clinical aspect Merritt and Fremont-Smith (1937), in a careful analysis of results obtained by the old method in 5,036 cases, reached the conclusion that mid and end zone curves were of no diagnostic significance, only *first* zone curves being specially related to disseminated sclerosis and neurosyphilis—the two conditions in which the test is usually considered to be of most value. First zone curves were, however, relatively infrequent in these diseases (32 per cent and 20 per cent respectively), and were considered of less significance in fluids with high protein content as they occurred occasionally in cases of purulent meningitis, cerebral haemorrhage and brain tumour.

While the significance of positive results by the buffer technique needs assessment on a larger number of cases, the results presented here suggest that first zone curves are of rather greater diagnostic importance than first zone curves obtained by the old method, because of the rarity of " false " positive reactions in conditions such as cerebral haemorrhage, purulent meningitis and the like. Moreover the proportion of first zone curves in neurosyphilis and disseminated sclerosis would appear to be higher with the buffer technique than with the old method. Thus of the 34 cases tested by the buffer method shown in Table III, 22 gave strong first zone curves (4 or 5+) and 5 gave weak first zone curves (2 or 3+). This gives 79 per cent of positive results as opposed to from 20 per

cent to 32 per cent of first zone curves in the two diseases in Merritt and Fremont-Smith's series.

In addition to this, fluid from 3 cases of neurosyphilis gave end zone curves with normal protein contents—a finding which appears to be diagnostically helpful. In this connection it may be noted that none of the protein fractions tested by Maclagan and Bunn (1946) with the buffer technique gave end zone curves, either singly or in combination. The chemical mechanism underlying this type of curve is therefore uncertain, but it presumably depends upon some property of the serum proteins which is abolished by electrophoretic separation.

SUMMARY.

1. A modified citrate method of preparing colloidal gold sols is described which is easier to reproduce than the oxalate method.

2. Buffer solutions are described which enable either the citrate or oxalate sols to be used for testing serum or cerebrospinal fluid without previous standardization. The serum technique at pH 7·7–7·8 is sensitive to 75 μg. of pure gamma globulin, while the cerebrospinal fluid method at pH 6·6–6·8 will detect 2 μg.

3. With the new technique only one tube is required for the serum test and only six for cerebrospinal fluid. With cerebrospinal fluid most of the positive curves are first zone in type.

4. Results with pathological cerebrospinal fluids show a high proportion of first zone curves in neurosyphilis and disseminated sclerosis (79 per cent), false positive results in other diseases being infrequent.

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AN ANTIBIOTIC FROM *BACTERIUM COLI*.

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A CONSIDERABLE literature has collected during the past 50 years on the antagonistic effects produced by *Bact. coli*. As a full review of these contributions will be published elsewhere, it is proposed to mention now only those considered the most important. In 1904 Eijkmann performed experiments which led him to conclude that several coliform bacteria produced thermolabile, inhibitory substances which were diffusible, but would not pass through a porcelain filter. He could not detect these inhibitors in broth cultures. Conradi and Kurpjuweit (1905) also claimed to demonstrate a similar heat-labile substance, though their remarkable statement that cultures could be diluted 3200 times and still stop bacterial growth seems hardly credible. Other authors such as Moro and Murath (1906), Leuchs (1907) and Chatterjee (1909) considered that *Bact. coli* elaborated a thermolabile antibiotic. Nissle (1916) published work on the "antagonistic index" of *Bact. coli* versus *Salm. typhi*. He thought that "active" strains produced something which inhibited the growth of a number of disease-producing bacteria. Active strains were sold under the name of "Mutaflor" for the treatment of various intestinal infections. McLeod and Govenlock (1921) found that broth filtrates obtained after the growth of *Bact. coli* would not support growth of *Bact. coli* nor certain other organisms, but if the culture media were diluted 1 part in 5 of water they again became capable of sustaining growth, thus the inhibition was not due to exhaustion of the medium. McLeod and Gordon (1922) showed fairly conclusively that a similar inhibitory effect produced by pneumococci was due to hydrogen peroxide formation. They found that some other organisms also formed hydrogen peroxide; though the strain of *Bact. coli* they examined in this respect did not do so, it seems quite possible that the strain studied by McLeod and Govenlock (1921) did produce hydrogen peroxide. Van der Reis (1921, 1922) described the destruction of *C. diphtheriae* by *Bact. coli* a thermolabile, non-dialysable and non-filtrable material being the active agent. Pesch and Zschocke (1922) also found that *Bact. coli* killed *C. diphtheriae*. Gundel and Habs (1927) and Gundel (1930) believed that *Bact. coli* produced thermolabile antibacterial substances, and Gundel (1930) extracted a "lipoid" from *Bact. coli* which was active against staphylococci and *Bact. coli*.

There are many other papers describing inhibitions produced by *Bact. coli*, but those of Gratia (1925 and 1932) are, from the point of view of the present paper, the most important. He noted that one strain of *Bact. coli* (called by him Coli V), which was very virulent for rabbits and guinea-pigs, produced something in the medium on which it grew which showed inhibitory action towards another *Bact. coli* strain (Coli ϕ) even when the medium was diluted as much as 1000 times. He deduced that as the active substance was freely diffusible through cellophane and was very stable, it was not a bacteriophage. It retained its

activity for months in sealed tubes, and withstood boiling for 1 hour and heating to 120° C. for half an hour. Later it was shown to be precipitated by acetone. The Coli V apparently lost for a time its capacity to produce the substance, but eventually recovered it spontaneously. A full account of his experiments was furnished by Gratia in 1932, when he illustrated the inhibition. He noted that although most of the Coli ϕ were inhibited around the colony of Coli V on an agar plate some resistant organisms grew up in the inhibited zone, and that though the development of Coli ϕ in broth might be stopped for some hours full growth eventually appeared. Thus there seemed to be bacteria of the Coli ϕ strain which were resistant to the Coli V substance. Gratia showed that the faster the Coli V grew the better the yield of the substance, and that if it was left in contact with the Coli V bacteria for 5 days it was destroyed. It had slight activity against *Sh. shigae*; whether any other organisms were tested is not stated.

Guelin (1943) described a substance (called L 36) very similar to that of Gratia. A special strain of *Bact. coli* was found to produce in the broth in which the organism had grown a substance which was thermostable and could be filtered. This substance was found to lyse very young cultures of *B. paradysenteriae* and another strain of *Bact. coli*, but resistant forms seemed to be readily produced. The material was not a bacteriophage. Calcium ions were apparently necessary for the development of the substance, but not for its lytic action.

Demelenne-Jaminon (1940) reported being able to separate two forms from the resistant strains of Coli ϕ of Gratia. One form grew in large colonies, 2–3 mm. in diameter, while the other formed minute colonies seen only with difficulty after 24 hours' incubation. On subculture both bred true for more than a year. Both had the characteristics of a true *Bact. coli*. The proportion of resistant bacteria in Coli ϕ was 0·004 to 0·005 per cent, and among these the large colony type was about twice as numerous as the small.

In 1945 Wiedling described the existence of a thermostable antifungal substance produced in fluid medium by a strain of *Bact. coli*. He was led to notice this by the fact that *Penicillium notatum* grew poorly on a medium which had been accidentally contaminated by *Bact. coli* (though there was no appreciable reduction of the nutrients). The inhibitory substance was not destroyed by autoclaving at 120° C. for 20 minutes. It was not isolated, nor were its properties further discussed.

A thermolabile lytic substance was observed by Zamenhof (1945) to be formed by a smooth strain of *Bact. coli*. This lysed specifically the cells of another rough strain, but was quite inactive against several other strains of *Bact. coli* as well as staphylococci and Gram-positive bacilli. The lytic substance was not a phage nor a specific polysaccharide. It was destroyed by heating for 15 minutes at 81° C., but was practically unaffected at 61° C. for 1 hour. It was stable for 1 month at 25° C. It was not bacteriostatic but bacteriolytic. The author stressed the high specificity of the action of this substance.

The immediate starting-point of the present work was the observation of Goldsworthy and Florey (1930) that a strain of *Bact. coli* obtained from cat faeces produced something which diffused through agar and inhibited the growth of *M. lysodeikticus*.

After this paper had been prepared for publication, it was learned (private communication to H. W. F.) that observations on highly specific inhibitions among

coliform and other bacteria have been made recently by Professor A. Gratia, Dr. P. Fredericq, and their colleagues. Several communications on the subject have been made to learned societies and though in process of publication, have not yet appeared. It is understood that the production of several different inhibitors (named " colicines ") by strains of *Bact. coli* has been proved, and that a system of classification is being worked out. Professor Gratia has kindly examined the *Bact. coli* strain CF 1 used in this work, and reports that it appears to be identical in all respects with his Coli V. To avoid later confusion, we are adopting his generic name "colicine" for our substance, but have otherwise not altered the text of this paper.

EXPERIMENTAL.

Although an examination of 200 strains of *Bact. coli* of human origin selected at random showed that seven of these produced inhibitors (Jennings, unpublished), the strain studied in the present paper was not isolated from human material, but, like that of Goldsworthy and Florey (1930), from cat faeces.

From the first specimen of faeces examined, one coliform organism was selected which on glucose agar gave good and quantitatively similar inhibition against *Staph. aureus*, *Bact. coli*, *B. subtilis*, *Ps. pyocyanea*, and *C. xerosis* (Fig. 1). On Lemco or heart agar, only *Bact. coli* was affected (Fig. 2). Further experiments strongly suggested that in the presence of glucose, hydrogen peroxide was formed, but that under certain other conditions, a more specific inhibitor appeared which was responsible for the selective inhibition of *Bact. coli*. The organism was called strain " CF1," and the specific inhibitor it forms is, as explained above, in this paper being called " colicine." The organism was identified* as an " atypical Type I *Bact. coli*," with the fermentation reactions shown in Table I.

TABLE I.—*Fermentation Reactions of* Bact. coli *Strain CF*1.

Lactose	Acid, gas	Indol	Positive
Glucose	,,	Litmus milk	Acid
Maltose	,,	Gelatin	Not liquefied
Mannitol	,,	Voges-Proskauer	Negative
Sucrose	Negative	Methyl red	Positive
Salicin	,,	Citrate utilization	Negative
Dulcitol	Acid, gas	MacConkey 37°	Acid, gas
Sorbitol	,,	,, 44°	—

Preliminary experiments.

Although a good inhibition was invariably obtained by the streaking technique on heart extract agar and Lemco agar with or without various additions, several attempts to obtain the inhibitor in liquid culture, with various media and under

* We are indebted for identification to Dr. Joan Taylor of the Emergency Public Health Laboratory Service, Oxford.

DESCRIPTION OF PLATE.

FIG. 1.—Streak of *Bact. coli* CF1 on glucose agar. Cross-streaked with 1 in 1000 dilutions of broth cultures of *Staph. aureus*, *Bact. coli*, *B. subtilis*, and *Ps. pyocyanea*, and with a 1 in 10 dilution of *C. xerosis*.

FIG. 2.—Streak of *Bact. coli* CF1 on heart agar. Cross-streaks same as in fig. 1.

FIG. 3.—For explanation see text.

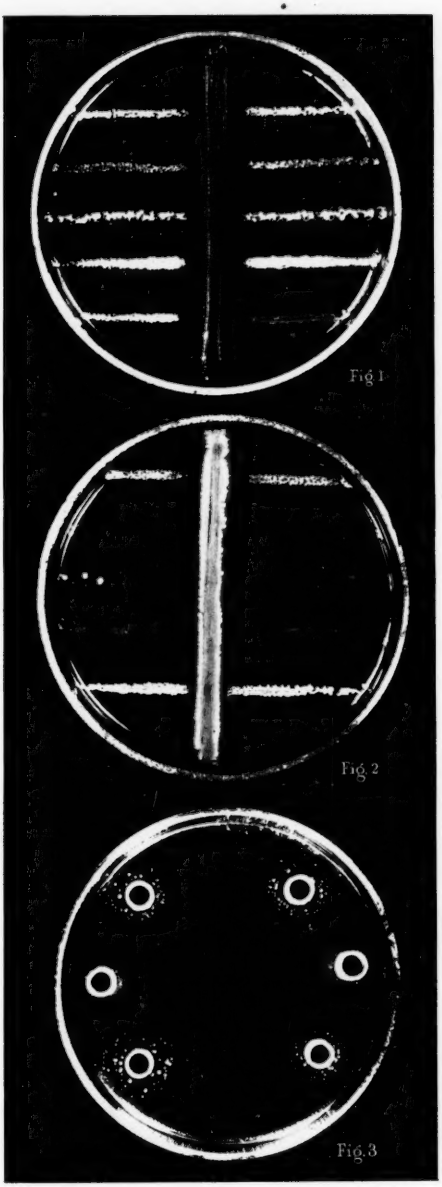

Fig 1

Fig 2

Fig 3

Heatley and Florey.

different conditions, were almost fruitless; at best only a trace of inhibition was obtained when the culture fluid was tested on a cylinder-plate. On the assumption that the formation of the inhibitor might require plentiful oxygen, the medium was used in thin layers or was shaken continuously, but little or no activity was produced. These liquid culture experiments were complicated by the fact that all viable organisms had to be removed from the liquid before testing, otherwise it was found that an inhibition might result from their growth during the test either in the fluid or on the agar inside the cup. In these unsuccessful tests this necessary sterility was achieved by centrifuging the suspensions, and then filtering the supernatant through a Gradocol membrane or a fritted glass filter. Seitz filtration and heating were not used because of the posssibility that the substance, whose properties were not known, might be adsorbed on the filter or be destroyed by heat. (Later, Seitz filtration was found to hold back almost all the activity of this substance.)

Selection of test organism.

In the streaking tests, the ordinary laboratory strain of *Bact. coli* appeared to be completely inhibited. When this same organism was used for seeding plates for cylinder-plate assays there appeared in the good zones of inhibition which were obtained with broth from cellophane sacs (see below), a few discrete resistant colonies (Fig. 3), similar in appearance to those described by Gratia (1925, 1932) and Demelenne-Jaminon (1940). The reason for this appeared to be that the original culture was not homogeneous in respect of its sensitivity to colicine, for plates seeded with a culture derived from one of the resistant colonies showed no inhibition at all, whereas plates seeded with cultures from other single colonies isolated from the original test *Bact. coli* gave zones of inhibition completely clear of resistant colonies (Gratia, 1932). One of these freshly isolated sensitive strains was used for all subsequent tests and assays, and over a period of several months no resistant colonies appeared.

Method of assay.

The cylinder-plate method (Heatley, 1944) was used, the plates being surface-seeded with a 1 in 1,000 dilution of the sensitive *Bact. coli* described below. The assay value was little affected by the density of seeding. Though sharp zones of inhibition were obtained, the curve relating zone diameter to concentration of colicine was rather flat, so that the accuracy to be expected was correspondingly less.

Production of Colicine.

Cellophane sac experiments.

Gratia (1925) fashioned a sheet of cellophane into the form of a bag, which was filled with broth and immersed in a crystallizing dish also containing broth. When the broth outside the sac was inoculated with his " V " strain of *Bact. coli* the active substance formed passed freely into the broth inside, which remained sterile. With this experiment in mind it was shown that strain CF1 would grow well on the surface of a sheet of sterile cellophane laid on the surface of an agar plate. The inhibitor passed through the cellophane and after the cellophane and the bacteria on its surface had been stripped off there was good inhibition of a sensitive organism planted on the surface of the agar. This technique has

also been described by Gratia (1944), though his paper did not come to hand till after the experiments described below had been completed.

Based on this discovery another attempt was made to obtain the inhibitor in a fluid medium, in order to facilitate extraction.

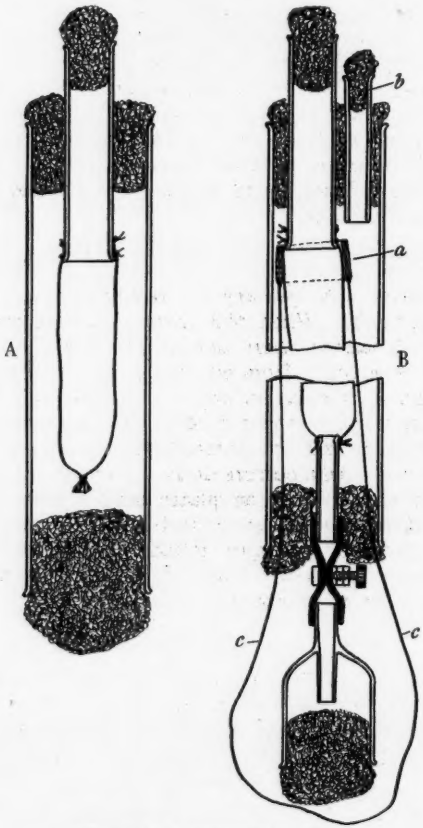

FIG. 4.—For explanation see text.

Cellophane sausage-casing sacs, tied onto glass tubes plugged with cotton-wool, were filled with Lemco broth and suspended in wide glass tubes plugged with cotton-wool at top and bottom (Fig. 4*A*). The whole assembly was sterilised by autoclaving, and when cool, the outside of the sac was inoculated with CF1 by stroking with a sterile swab dipped into a broth culture of the organism, the swab being introduced at the bottom of the tube after removal of the cotton plug.

After 24 hours' incubation at 37° C., the sterile broth inside the sac invariably gave a well-defined zone of inhibition of 16–18 mm. diameter on a cylinder-plate.

The medium could be replaced at this stage by fresh medium, and after 24 hours' further incubation (and without any further seeding or interference with the bacteria on the outside of the sac) another crop of active broth could be harvested. This replacement could be repeated many times with no apparent alteration in the yield of inhibitor, and it afforded an easy way of testing different media by putting in the sac, first broth, then a test medium, then broth again, then another test medium, and so on. Several modifications of the cellophane sac set-up were devised.

E.g., a tube tied into the bottom of the sac for withdrawing medium is shown in Fig. 4B, which also shows the arrangement used for sowing long sacs. After sterilising, the cloth cuff *a* is moistened with a few drops of broth culture (introduced through the small tube *b*), then slowly pulled down the length of the sac by means of two threads *c* passing through the plug at the bottom of the tube. After pulling out, the threads are cut off close to the plug and touched with a drop of lysol. An arrangement by which medium was continually allowed to drip into the top of the sac and run off from the bottom at the same rate through an automatic syphon was not found to have any advantages over the static arrangement, and was certainly more trouble.

Attempts to increase the scale, e.g. by suspending many sacs in a large bin, or laying them flat in trays, and seeding by means of a spray, were not very satisfactory, but it is worth mentioning that sacs filled with broth and tied off at both ends could be autoclaved without bursting and without much loss of broth.

A more practical method of increasing the scale was to cover a thin layer of broth in an enamel tray with cellophane, pleated so that the whole surface of the broth was covered, and extending well over the edges of the tray. The upper surface of the cellophane, which was sown with bacteria by a spray, was protected from contamination by a single thickness of lint secured by a draw-string. Stretched zigzag wires clipped over the edges of the trays supported the lint and prevented it from sagging unduly. The unwetted parts of the cellophane became brittle after autoclaving, but tearing during harvesting and replacement of the medium were avoided by directing a jet of steam onto the area of the cellophane which was to be unfolded.

Production of inhibitor on media other than Lemco.

Various adjuvants to the plain Lemco broth were tried in cellophane sac experiments in the hope of increasing the yield, but the addition of Marmite, corn steep liquor, gelatine digest, casein digest, lactose, lactate, extra peptone, extra Lemco, or extra glucose, made no appreciable difference. Dilution of the broth with water lowered the yield. Heart extract broth gave about the same yield as Lemco broth. Various synthetic media were tried. When the plate streaking test was carried out on a synthetic agar containing only glucose, KH_2PO_4, $MgSO_4$ and ammonium sulphate or asparagine, good inhibition against several different bacteria was obtained (presumably due to hydrogen peroxide), but the same medium in sacs gave no inhibition at all, even against the sensitive *Bact. coli*. It was noted that in Lemco and heart extract broth, in which activity

was regularly obtained, the pH was always above 8·0, whereas that of the synthetic media was at or below 6·5. Addition of alkali, buffer, calcium carbonate or magnesium trisilicate to various media, during or at the beginning of an experiment, and with continuous agitation in the case of the solid reagents, gave no activity. When lactate was substituted for glucose with the object of raising the pH during fermentation, a definite, though small yield was obtained on three occasions, but after this could not be repeated, though several tests were made and a number of different samples of lactic acid were tried.

Aeration experiments.

It was puzzling that a good yield of inhibitor should be obtained when the bacteria were separated from the medium, whilst little or none was obtained when the bacteria were actually grown in it—especially so when it was found that prolonged incubation of a heavy suspension of living CF1 bacteria with a concentrate of colicine, followed by heat killing of the cells, did not diminish the activity at all. The experiences with cellophane again brought up the idea that proper aeration might be the key to the problem, although the results of early experiments with shaken cultures in thin layers of medium did not support it. The idea was fruitful, for when much more intensive aeration was produced by blowing a vigorous stream of air through inoculated Lemco broth, a good yield of inhibitor was formed—approximately the same as that obtained in cellophane sacs. Samples of these aerated broths were before assay placed in a closed phial, and completely immersed in a boiling water bath for about 5 minutes to kill the bacteria. It was shown that even much longer heating did not diminish the antibacterial activity.

Routine production.

The final procedure adopted for the production of colicine was the following :

Into rectangular porcelain vessels of the type used in this laboratory for the production of penicillin (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941), were placed two litres of nutrient broth of the following composition :

Lab. Lemco	10 g.
Bacteriological peptone (Eupeptone or from Parke, Davis & Co.)	10 g.
Tap water	1 litre

The pH was adjusted to 7·0–7·2, and the liquid steamed or boiled for 30 minutes, then filtered. The pH was then adjusted to 6·8.

1–2 ml. of lard oil or arachis oil were added to each vessel to prevent foaming. The vessels, which were kept upright throughout, were closed by a cotton-wool plug through which a glass tube passed to the bottom of the vessel ; the outer end of the tube was wrapped in lint or paper. The vessels were autoclaved for 30 minutes at 1 atmosphere pressure, then transferred to a water bath at 37° C. They were connected, with aseptic precautions,

to a separately sterilised train comprising (*a*) an air steriliser (a glass tube 3 cm. in diameter, by 40–50 cm. long, tightly packed with non-absorbent cotton-wool) connected to (*b*) a manifold, from the side tubes of which connection was made with (*c*) wash bottles containing water and a little oil. The outlet tubes of these wash bottles were wrapped in lint during the sterilising, and were subsequently connected, with aseptic precautions, to the vessels. The wash bottles also were immersed in the water bath and served two purposes, namely, moistening the air blown through the vessels, which prevented much evaporation from the latter, and providing a guide to the rate of aeration.

The vessels were seeded by tipping into each a small 24-hour Lemco broth culture of CF1, and aeration was begun, the stream of air being as fast as the wash bottles would allow. After about 16 hours the vessels were disconnected and steamed in the autoclave for 10–15 minutes to kill the bacteria, which were removed by passing the broth through a Sharples centrifuge.

Extraction.

From the clear broth, of which the pH was usually about 8·5, the inhibitor was removed by shaking with two successive portions of 0·5 per cent and 0·3 per cent respectively of " Farnell Grade 14 " charcoal, which were separated on Buchner funnels, with the help of Celite. One-half to one-quarter of the amount of " Darco G 60 " or " Puractim M " charcoal was as efficient as the amount and grade of charcoal actually used, but at that time these more effective grades were not obtainable in bulk.

The charcoal residues were briefly washed with water whilst on the funnels, then were boiled for a few minutes with 80 per cent alcohol, which removed a certain amount of yellowish inactive substance. This alcohol washing was usually repeated, then the charcoal was spread out to dry. The inhibitor was eluted from it with glacial acetic acid, which, apart from phenol and possibly pyridine, was the only effective eluent of the many which were tried. The charcoal and glacial acetic acid were mixed to a thick cream, then poured on to a Buchner funnel to which suction was applied for about half an hour, whilst at intervals the cake was firmly pressed down to obliterate cracks. Suction was stopped and more acetic acid was poured on to the cake, which slowly imbibed it. After a few minutes suction was again applied for half an hour, then the whole cycle was repeated three or four times, or until the filtrate was only faintly coloured. Usually the second, presumably weaker, batch of charcoal was eluted first, and the filtrate from this used for the elution of the first batch. The eluates were concentrated to a syrup by vacuum distillation, preferably in separate batches so that prolonged heating of any of the material was avoided. The syrup was ground up with sand and a large volume of absolute alcohol was gradually added. The resulting flocculent precipitate did not settle as readily as the sand, and could be poured off, with alcohol washings, into centrifuge tubes, and spun down. The alcoholic supernatant, carrying most of the acetic acid, was poured off, and the precipitate well stirred and shaken with fresh alcohol, then again spun down. The washing was repeated once more, then the precipitate (which seemed to have thixotropic properties) was transferred to a dish and dried in a

thin layer at 37° C. In one experiment, 40 litres of crude broth yielded in this way 23 g. of dirty white non-hygroscopic chalky solid.

The solid was stirred and shaken with water, of which the pH was adjusted to 8·0–8·5, and then centrifuged. After washing twice more with water at the same pH, the solid was inactive and was discarded. The strongest watery extract was treated with a small volume of phenol—perhaps 1 per cent—and thoroughly shaken. An opalescence occurred, but if this did not develop into an easily centrifugable precipitate within half an hour or so, more phenol was added and the shaking was repeated. When a definite precipitate formed, the fluid was centrifuged, the supernatant was poured off and the active residue drained as fully as possible. From the supernatant, more active precipitate could be obtained by adding more phenol, but these subsequent crops were brown and semi-fluid, whereas the first was flocculent and pale cream in colour. The final supernatant contained much inactive material and was discarded. The various less active watery extracts and fractions were incorporated at suitable stages in the next batch to be worked up.

The first phenol precipitate was stirred up with water at pH 8·0, and shaken with an excess of ether, which removed the phenol. The watery solution sometimes threw down a precipitate on standing, which contained some activity, though most remained in the clear faint brown supernatant. This could be dried in a desiccator to a brittle glistening transparent brown non-hygroscopic glaze. Yield, 10–20 mg. per litre of crude broth. It was this material which was used for most of the animal experiments and bacteriological work.

PROPERTIES.

General properties.

The material is freely soluble in aqueous solutions at any pH, but is insoluble in all the usual organic solvents except acetic acid, and, to a small extent, pyridine; it is slightly soluble in moist phenol, for it can be eluted from charcoal with this reagent, although it is also precipitated by it from strong aqueous solution. It is odourless and has only a faint taste reminiscent of gelatine. On heating gradually on a slide it bubbles slightly, turns brown and then black, and gives a smell typical of burning protein. It is freely dialysable through cellophane. Solutions have a strong tendency to foam, and even with almost continuous addition of caprylic alcohol it was not possible to concentrate the material by distillation *in vacuo*. Foam separation was attempted, with a slow stream of compressed air as generator of foam, but although there was no destruction of colicine, there was no apparent concentration of it, either in the foam or mother liquor, when tests were carried out at pH 4·6, 5·0, 5·2 or 8·3. The active substance appears to be of a peptide nature; it is hoped to report on this at a future date.

Stability.

The activity is not appreciably reduced by boiling for half an hour at pH 2·0 or 7·0, but about 75 per cent of the activity is destroyed in half an hour at 100° C. and pH 9·0. No detectable activity remains after boiling for 25 minutes with N/2 hydrochloric acid or N/10 sodium hydroxide. The aqueous solution appears to keep its activity unimpaired in the ice chest for several months.

Destruction by enzymes.

Colicine is destroyed almost immediately by commercial pepsin or commercial trypsin. It is destroyed fairly rapidly by slices of mouse kidney, and more slowly by mouse liver. The supernatant from a centrifuged sample of staphylococcal pus slowly destroyed the activity of the substance when incubated with it.

Antibacterial properties.

Effect of inoculum size.—The diameter of the zone of inhibition in the cylinder-plate assay varied little with differences in the density of seeding of the plates, over a thousandfold range. A good dried preparation gave a zone of inhibition of *about* 18 mm. diameter at a dilution of 1 in 40,000. In broth culture, however, the titre was markedly affected by the size of inoculum, as shown in Table II.

TABLE II.—*Relation of Inoculum Size to End-point.*

Two-fold dilutions in Lemco broth. Test organism: sensitive *Bact. coli.* Same sample of colicine used in all experiments.

Experiment No.	Dilution just preventing growth; 1 part in . . . million.		Inoculum size. Viable organisms per ml.
	After 24 hours.	After 48 hours.	
1	32	32	750
2	12·8	12·8	660
3	8	4	2,800
4*a*	6·4	1·6	4,000
b	6·4	6·4	,,
c	3·2	3·2	,,
d	6·4	3·2	,,
5	6·4	6·4	approx. 2,000
6	3·2	3·2	15,000
7	2	1	approx. 400,000
8	0·05	0·05	15,000,000

Effect of serum.

The addition of up to 50 per cent serum with or without preliminary incubation made no noticeable difference to cylinder-plate assay values, but in dilution tests the presence of rather small amounts of serum definitely enhanced the potency of the substance, as shown in Table III. This effect did not appear to be related

TABLE III.—*Effect of Serum on End-point in Lemco Broth against Sensitive* Bact. coli.

Twofold dilutions.

Experiment No.	Inoculum size. Viable cells/ml.	Highest dilution just preventing growth. 1 in . . . million.		Final concentration of serum.
		In plain Lemco.	In Lemco + serum.	
1	—	6·4	25	10%
2	750	32	64	20%
3*a*	2,800	4	128	20%
3*b*	280,000	0·5	16	20%
4	—	2	8	10%

to pH, for the same end-point was obtained in plain Lemco broth whether the latter was buffered to pH 7·0 or to pH 7·4 (Abraham and Duthie, 1946). Serum heated to 56° C. for 30 minutes was as effective as raw serum.

Range of bacteria attacked.

In view of the marked effect of the inoculum size on the titre in broth, dilution tests on a variety of different organisms were not carried out. Instead, some idea of the relative sensitivity of different species was obtained from semi-quantitative gutter-plate experiments. Gutter plates were made containing graded concentrations of colicine in the gutter, and the various organisms being investigated were streaked across the plate perpendicular to, and on the surface of, the gutter itself. Such an experiment showed that a preparation which would inhibit the test *Bact. coli* for a distance of 8–10 mm. from the edge of the gutter could be diluted between 1600 and 3200 times before the same test organism would actually grow across the gutter. The following organisms were found to be at least 1000 times more resistant (possibly much more so) than the test *Bact. coli;*

Bact. coli N.C.T.C. No. 86 ; *Salm. typhi* (2 strains) ; *Bact. coli* strain CF1 ; *Sh. flexneri ; C. xerosis : Myco. phlei :* El Tor vibrio *: Ps. pyocyanea* ; *Salm. typhimurium* ; *Salm. newport.*

Sh. shigae and *Sh. sonnei* were approximately as sensitive as the test *Bact. coli.*

Salm. enteritidis was about 60 times and *Salm. paratyphi A* about 100 times more resistant.

Mode of action on bacteria.—Sensitive bacteria are killed, not merely inhibited, but lysis has not been observed, even after prolonged incubation of sensitive and insensitive strains of bacteria in a solution containing 1 per cent of colicine. Incubation of cells from a young culture of sensitive *Bact. coli* in a 1 in 6000 solution of the substance in broth was not attended by any change in turbidity, and the nature of the zones of inhibition in the cylinder-plate test is what might be expected from a slowly diffusible non-lytic substance.

Sensitivity of pathogenic Bact. coli.—Nineteen strains of *Bact. coli* isolated from cases of bladder infection were tested for sensitivity to colicine by the streaking test. Seven were definitely inhibited, with nine there was a trace of inhibition, and with three none at all.

Action on red blood corpuscles.—Incubation of human red cells with a 1 in 100 solution of colicine in normal saline for 20 hours was accompanied by no more haemolysis than in saline alone.

Action of bacteria on colicine.—A solution of partially purified colicine was incubated at 37° C. for several hours with thick suspensions of living *Bact. coli.* The suspensions, which were heat-sterilised before re-assay, showed no diminution of activity with any of the strains tested, whether they were susceptible to the action of colicine or not.

Pharmacological properties.

Toxicity to mice.—Mice weighing 19 or 20 g. were injected intravenously with colicine dissolved in saline. Usually doses of 4 or 5 mg. (5 mice) had no effect ; 8 or 10 mg. (3 mice) produced slight sickness with recovery after a few hours, and 18 mg. (1 mouse) also produced slight sickness. There was an exception,

however, with one batch of material, of which doses of 5, 10 and 20 mg. respectively to 3 mice produced increasing sickness and death in 8 hours or more, though 2·5 mg. had no effect.

Toxicity to leucocytes in vitro.*—Human leucocytes were suspended in a solution containing colicine by the method previously described (Abraham *et al.*, 1941), and were watched for some hours. In 1 in 1000, the strongest concentration tested, the cells were as active throughout as in the control preparations.

Effect on smooth muscle.—Colicine was added to the Ringer-Locke solution in which was suspended the rhythmically contracting isolated horn of a guinea-pig uterus. No substantial effect on the contractions was produced by a concentration of 1 in 5000, the highest concentration tested.

Excretion.—Five mg. was injected intravenously into each of 2 mice, weighing respectively 23 and 25 g. The mice were killed 5 hours later and the urine tested on cylinder-plates. There was no activity. In another similar experiment, when 18·5 mg. were given, the urine gave a small but definite zone of inhibition, but the amount excreted was less than 5 per cent of the dose given. The material seems to have been largely destroyed in the body, which observation agrees with the results of the tissue slice experiments.

SUMMARY AND DISCUSSION.

That a variety of different bacterial inhibitors are formed by different strains of *Bact. coli* is evident from the differences in their reported heat stability, diffusibility, nature of action (lytic, bacteriostatic or bactericidal) and by the range of bacteria attacked. It is probable that in some cases the active agent is hydrogen peroxide. The strain forming the subject of this paper, strain CF1, an atypical *Bact. coli* Type I isolated from cat faeces, forms hydrogen peroxide under some conditions, but also a more specific inhibitor, possibly of a peptide nature, which appears to be different from those already reported as being formed by various strains of *Bact. coli*. The substance (called colicine) was formed under conditions of good aeration in Lemco or heart extract broth, but not in any of the synthetic media which were tried. At first it could only be demonstrated on a solid medium (Eijkmann, 1904; Foster and Woodruff, 1946), but later a good yield was obtained in broth if the culture was vigorously aerated. Foster and Woodruff had a similar experience with bacillin, formed by a *B. subtilis*, but in their case it was the addition of manganese to the medium, not aeration, which made production in fluid medium possible.

Colicine is not lytic, but it will kill a small inoculum of a sensitive organism at a dilution of 1 in several millions, though the inoculum size has a very considerable effect on the titre. In its action it is extremely specific, some strains of *Bact. coli* being among the most sensitive, other strains being unaffected. In this respect it resembles the specific lytic substance of Zamenhof (1945), but is sharply distinguished from it by its heat-stability and bactericidal action. (A preliminary investigation suggests that there may be many other inhibitors which are equally specific, formed by other strains of *Bact. coli*. These facts reinforce the feeling that must have been shared by other workers, that in the detection of antibiosis, much will depend on the choice of the test organisms—a choice hitherto guided largely by tradition or chance, and for which there is as

* Experiment done by Dr. M. A. Jennings.

yet no rational basis. Clearly an antibiotic as specific as colicine could easily escape detection if only a few test organisms were used.)

The potency of colicine is enhanced by serum, an effect which is believed to have been observed so far only in the case of gramicidin (Dubos, in press) and of an inhibitor formed by *B. subtilis* (Abraham and Gilliver, personal communication). Almost nothing is yet known of its chemical nature, except that it is freely soluble in water and behaves like a peptide, and that it is precipitated from concentrated crude aqueous solution by phenol. Impure as the dried product which has been obtained undoubtedly is, it has only a moderate toxicity to mice, and a remarkably low toxicity to leucocytes. It is non-haemolytic.

The substance is unlikely to be of any chemotherapeutic value as it is fairly rapidly destroyed in the body, and is also destroyed by at least some kinds of pus. It is most improbable that *Bact. coli* strain CF1 through its elaboration of colicine can exert any effect on the intestinal flora, as was postulated for some strains of *Bact. coli* by Nissle (1916), both because a liberal supply of oxygen appears to be necessary for the formation of the antibiotic, and also because the latter is destroyed almost immediately by trypsin and pepsin.

ACKNOWLEDGMENTS.

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THE PRESERVATION OF MAMMARY TUMOUR AGENT BY DESICCATION OF THE BREAST TUMOUR TISSUE OF MICE.

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THE lack of a stable preparation of the mouse mammary tumour agent greatly increases the difficulties which are encountered during the investigations of the agent. Since there is no standard of mammary tumour agent, each breast tumour extract has to be tested separately for its activity. The longest survival of the agent, so far recorded, in dried tumour tissue is six months (Bittner, 1944*a*). Preservation of the agent for periods longer than that reported by Bittner, should it be found possible, would greatly help in standardization of the material employed as a source of the agent.

In previous experiments (Dmochowski, 1944, 1945*a*, *b*) mouse breast tumour tissue of various high cancer strains, dried in vacuum over phosphorus pentoxide, was used as a source of the agent. The dried tumour tissue, stored in the ice chest for periods varying from 2 to 5 weeks, was active and induced a high incidence of breast cancer in susceptible mice. The tumour-producing potency of R III strain dried breast tumour tissue was found to be greater than that of other high cancer strains of mice.

Following these findings the present experiments were carried out to ascertain whether the mammary tumour agent can survive in dried breast tumour tissue for periods longer than those so far recorded, and still induce a high incidence of breast cancer in susceptible mice.

METHODS.

C 57 × R III hybrid mice, born and raised in the laboratories, were employed in the experiments as test mice, since previous observations indicated that these mice are susceptible to the development of breast cancer. In females of this hybrid strain breast cancer has not so far been observed to develop spontaneously.

R III breast tumour tissue was used as the source of the mammary tumour agent. A number of tumours which arose spontaneously in R III strain females were minced together and desiccated in a vacuum chamber over phosphorus pentoxide. The air was evacuated by means of a Cenco-Hyvac pump which was left to run for twelve hours. The breast tumour tissue was kept in the vacuum chamber for an additional period of 48 hours and then put into vials from which air was evacuated, and stored for 24 months in the ice chest.

Before use, the tissue was resuspended in distilled water in a dilution of 1 : 10 and injected subcutaneously into the mice. Each mouse was given, at each injection, 0·5 c.c. of the suspension containing approximately 0·05 g. of breast tumour tissue. The injections were given at two days' intervals. Each mouse received 16 injections comprising altogether 0·8 g. of breast tumour tissue.

The C 57 × R III hybrid females of each litter, when 4 to 5 weeks of age,

were marked individually and divided as equally as possible into experimental and control mice. All females were forcibly bred by removing each litter within 24 hours after birth, and were kept under similar conditions on a diet of "rat-cake" cubes with an unlimited supply of tap-water.

RESULTS.

The results of the experiment are summarized in Table I.

TABLE I.—*Appearance of Breast Tumours in forcibly bred* C 57 × *R III Female Mice after administration of R III Dried Tumour Tissue, Stored for* 24 *months in the Ice Chest.*

Number of mice.	Age of mice in weeks.	Number of injections.	Number of breast tumours appearing monthly.							Tumours.		Average tumour age in months.
			6	7	8	9	10	11	12	Number.	Per cent.	
23	4–5	16	2	3	5	7	3	1	1	22	96	8·5

No tumours developed in any of the 20 litter mate control females which were forcibly bred but not injected with R III dried breast tumour tissue.

As can be seen from Table I, dried R III breast tumour tissue stored in evacuated vials in the ice chest for 24 months induced a high incidence of breast cancer at a comparatively low average tumour age. The mammary tumour agent, therefore, retained its tumour-producing potency in the R III dried breast tumour tissue for a period longer than any so far recorded. The tumour incidence was similar to that which was induced in susceptible hybrid females by the same R III dried breast tumour tissue stored in the ice chest for a period of three weeks.

DISCUSSION.

Bittner (1941) found that lyophilized breast tumour tissue resuspended in saline induced breast cancer in mice which were fed on it. Addition of 50 per cent glycerine to extracts of breast tumour tissue preserved their activity at ice chest temperature for 7 to 9 days. These glycerolated extracts were tested by feeding or intraperitoneal injections (Bittner, 1942). Bittner (1944*a*) also reported that dried breast tumour tissue, when fed to susceptible mice, was still active six months, but lost all its potency twelve months after desiccation. According to Bittner (1944*b*) lactating mammary gland tissue desiccated at room temperature lost all its activity within a week. Andervont and Bryan (1944) found that the mammary tumour tissue kept in a 50 per cent glycerine saline solution for seven days at 8° C. preserves the activity of the agent. This material is inactive, however, when fed to mice 80 days after glycerolation.

The present experiments have shown that R III breast tumour tissue dried under the conditions described was still active after 24 months' storage in the ice chest. The difference between the results obtained during the course of the present investigations and those of previous workers may be due to the greater activity of the original material as compared with that of the material employed by other workers, to the method of storage in evacuated vials and, perhaps, although to a lesser degree, to the mode of extraction and administration of the dried tissue. It was shown previously (Dmochowski, 1945*a*, *b*) that there exist differences between the tumour-producing activity of dried breast tumour tissue

derived from various high cancer strains of mice. If such differences exist between the tumour-producing potency of the agent in mammary tumour tissues of various high cancer strains of mice maintained in one laboratory, they probably will also be found in breast tumour tissue of the same high cancer strain bred in various laboratories. It cannot be stated whether the storage in evacuated vials influenced the activity of the dried material, as no comparison was made with the dried material stored in other ways. The method of storage, however, employed by Bittner (1941) was similar to that employed in the present experiments. The extraction with distilled water as compared with saline extraction employed by other workers may be more efficient and, at least to a small extent, account for the result obtained. This, however, cannot be stated for certain, as no comparable extraction with saline and distilled water of the same material was carried out. The mode of administration of the resuspended dried material may also have influenced the final result. In the present experiments desiccated material resuspended in distilled water was given in repeated subcutaneous injections, while previous workers mostly employed the method of feeding mice. This latter method, although effective with the fresh and the desiccated tissue kept for comparatively short periods of time, may not be the method of choice in testing the material for loss of activity which occurs during the storage.

It is difficult to show whether any loss of activity of the material occurred during the 24 months' storage in the ice chest. The original material when stored for only three weeks induced a similar incidence of breast cancer in susceptible C 57 × Strong A hybrid females when given in a smaller dose (0·2 g.) by four subcutaneous injections. These two results cannot, however, be compared, because C 57 × Strong A hybrid females may differ from C 57 × R III females in their susceptibility to tumour induction, and a minimum effective dose or at least equal dose was not used.

The results may prove to be useful in future experiments with the mammary tumour agent by removing the necessity of a constant supply of fresh tumour material during the long lasting experiments, and may be helpful in the standardization of the breast tumour material for studies of the agent.

SUMMARY.

A high incidence (96 per cent) of breast cancer was induced by injecting R III breast tumour tissue, dried and stored in the ice chest for 24 months, into 4 to 5 weeks old susceptible (C 57 × R III) hybrid female mice which were forcibly bred.

It is suggested that this may be helpful in obtaining a standard material for experiments with the mammary tumour agent.

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IMMUNITY TO ANTHRAX: PROTECTIVE ANTIGEN PRESENT IN CELL-FREE CULTURE FILTRATES.

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EXPERIMENTS recorded in this paper show that a highly effective immunizing antigen is produced extracellularly in cultures of *B. anthracis* under certain conditions. The antigen has very similar properties to those of anthrax oedema fluid, which has been studied recently by Grabar and Staub in France and Cromartie and his colleagues in America. Although Bail's (1904) original observation on the immunity produced by oedema fluid has been confirmed many times (Okuda, 1923; Matsumoto, 1924; Hruska, 1926; Urbain and Rossi, 1926, 1927; Stamatin and Stamatin, 1936; Ivánovics, 1938, and others), it is only recently that the nature of the active component has been investigated.

Staub and Grabar (1944) Grabar and Staub (1944) showed that oedema fluid contained two polysaccharides *a* and *b* both free and united with protein; *b* was immunologically identical with that found by Ivánovics (1939) in the organism. Since both were precipitated by the euglobulin fraction of immune serum, which did not contain the protective antibodies (Grabar and Staub, 1942), they at first considered that they were unconnected with the protective antigen. Later, however (Grabar and Staub, 1946), they successfully immunized guinea-pigs with that fraction of oedema fluid which contained polysaccharide *a*. The activity of this fraction was removed by precipitation with the euglobulin of immune serum or by the euglobulin fraction previously absorbed with Ivánovics' polysaccharide *b*. The isolated polysaccharides were inactive. They concluded that the active fraction was probably a combination of polysaccharide *a* and a protein.

Cromartie, Watson, Bloom and Heckly (1946) prepared oedema fluid in rabbits capable of protecting rabbits against 10,000 A.L.D. of spores with three weekly doses of 0·5–1·0 ml. The crude fluid was toxic, and gave rise to well-marked skin lesions in rabbits when injected intracutaneously. The toxic factor, however, was distinct from the immunizing agent and could be removed by adsorption on calcium phosphate (Watson, Cromartie, Bloom, Kegeles and Heckly, 1946). Chemical and physical investigation of the protective antigen after removal of the toxic factor showed that it was destroyed at 57° C. for 30 minutes and by trypsin and was probably protein in nature. Attempts to concentrate it by chemical means were not very successful. There was evidence that the bulk was present in the protein precipitate from 25 and 40 per cent ethanol. Electrophoresis experiments showed that it migrated between the β and γ globulins. There was not, however, a well-marked boundary, activity being present over a rather wide zone. The crude oedema fluid was also used to immunize other

animals. It was effective in sheep against 100 A.L.D., although not completely so. Partial protection was also obtained in guinea-pigs against 10 A.L.D. Doubtful results were obtained in mice. This is in agreement with most other workers (Ivánovics, 1938).

The question arose as to whether this antigen could be produced *in vitro*. Numerous attempts have been made to produce active culture filtrates with indifferent success (Sobernheim, 1913). In the light of results recorded below, two papers are worthy of mention. Casagrandi (1900) claimed to have produced some immunity in rabbits, with 30-day culture filtrates containing 1 per cent alkaline egg albumen. Later (1902) he used 50 per cent plasma cultures in broth. Large doses were given and the immunity was not of a high order. More recently Schilling (1927) used culture filtrates containing 10–100 per cent bovine or horse serum. He grew the cultures for 2–14 days and tested the filtrates in doses of 2–10 ml. in rabbits and obtained on the average 50 per cent survival. Unfortunately, the strain he used for challenge barely killed his controls in 8 days with 1 ml. broth culture. He himself concludes that no high degree of resistance was obtained. His results, however, are of some significance, since filtrates of cultures not containing serum were completely inactive. On the other hand, Basset (1933) claimed as good a result with peptone broth cultures without serum.

Recently White (1946*b*) has obtained some immunity in guinea-pigs using a non-sporing variant of the " Vollum " strain grown in whole blood, the whole culture killed with toluene being used as the vaccine.

The present paper gives the conditions for producing cell-free antigen in culture filtrates quite as active as that in oedema fluid.

TECHNICAL DETAILS.

Strains of B. anthracis *used.*

These were as follows: (1) " Vollum." This was a highly virulent strain obtained from Dr. Vollum of Oxford. The approximate A.L.D. was: for mice (intraperitoneal injection) 300 spores; for rabbits and guinea-pigs (intracutaneously or subcutaneously) 1,000 spores; for sheep* (subcutaneously) 800 spores, and for monkeys* (subcutaneously) 100,000 spores. (2) " M36." This was the " Vollum " strain after passage through monkeys, obtained from Dr. D. W. Henderson. The virulence for all animals was increased (Henderson, personal communication). (3) Strain " Hagan " from Dr. W. A. Hagan of Cornell University. This was also a virulent strain, but less so than " Vollum " or " M36." (4) " Weybridge " from the Ministry of Agriculture, Weybridge. This was an avirulent non-capsule forming strain used in the preparation of spore vaccines. (5) " HM." This was a mucoid variant of " Vollum " obtained from Dr. P. Bruce White of the National Institute for Medical Research, Hampstead. It was avirulent and did not form spores (White, 1946*a*). In addition, the following strains were obtained from the National Collection of Type Cultures: N.C.T.C. 109, 1711, 2620 and 5444. N.C.T.C. 1711 was Pasteur *premier vaccin* and 5444 was listed as avirulent. No details as to the virulence of the other

* With sheep and monkeys no accurate information could be obtained as to the A.L.D. owing to the few animals available. Of 2 sheep injected with 88 spores, both survived; of 3 given 880 spores, all succumbed in 5–7 days. One monkey given 10^4 spores survived, two given 10^5 died in 8 and 9 days, and one given 10^6 died in $2\frac{1}{2}$ days.

strains were available. Strains " Hagan " and " M36 " were the strains " C.D. 2 " and " C.D. 25 " of Cromartie, Bloom and Watson (1946).

Most of the work was done with the " Vollum " strain. Unless otherwise stated, this strain was used.

Spore Suspensions.

Except with the non-sporing strains N.C.T.C. 1711 and " HM " spore suspensions were used both for inoculating cultures and as a challenge dose in testing the efficacy of the vaccine. These were prepared as follows: The growth from 42-hour cultures on casein hydrolysate-yeast extract (CCY) agar (Gladstone and Fildes, 1940) was scraped off and suspended in distilled water and heated at 60° C. for $1\frac{1}{2}$ hours. Viable counts were carried out and the suspensions stored in the ice-chest in concentrations of 2–3 $\times$ 10^{10} spores/ml. The same spore suspensions of " Vollum " and " Weybridge " were used throughout the work. Viability and virulence (of " Vollum " strain) were tested at intervals. No fall in either was found.

Vegetative Suspensions.

With the non-sporing strains " HM " and N.C.T.C. 1711, 24-hour CCY agar cultures were used. The growth was washed off in saline and used directly.

Capsulated suspensions of " Vollum " were also used. The method used for their preparation, which was worked out with the co-operation of Lord Stamp, made use of the fact that capsules are formed when *B. anthracis* is grown in an atmosphere containing high concentrations of CO_2 (Nungester, 1929; Sterne, 1937). Cultures were grown in CCY broth in an apparatus in which the medium (2 l.) was continually aërated with 500 ml./min. of air containing 10 per cent CO_2 finely distributed by means of a glass sinter disc. In 18 hours a heavy growth of organisms showing 100 per cent capsulation was obtained. The culture was centrifuged and an approximate standardization of quantity was made, using vaccine opacity standard suspensions.

Preparation of Culture Filtrates.

Culture vessels.

For liquid media, at first these were 100 ml. Erlenmeyer flasks. Later, and for all solid media, 8-oz. " medical flats " were used laid flat. Each contained 25 ml. medium. For larger cultures 32-oz. bottles were used containing 100 ml. medium.

Inoculum.

Dilutions of the suspensions described above were made just before use in tryptic digest of meat broth, and a known number of spores or known opacity of vegetative culture in 1·0 ml. inoculated per 25 ml. medium.

Constituents of the media.

A large number of different media were investigated. These included media made with filtered extracts of rabbit organs, muscle and skin, leucocytes and red cells and oedema fluid produced by injection of turpentine. As there was no

indication of any activity in filtrates of such cultures, the details of their preparation need not be given.

Broth.—This was either the casein hydrolysate yeast extract (CCY), or tryptic digest of beef (TMB).

Serum.—Blood was collected from the jugular veins of rabbits and sheep into agar-lined centrifuge pots. The clot was allowed to contract and the clear serum centrifuged and Seitz filtered.

Plasma.—Rabbits and guinea-pigs were anaesthetized, the thorax opened and blood removed from the heart under aseptic precautions with a bulb Pasteur pipette. 73 ml. blood was mixed with 27 ml. 2·5 per cent sterile sodium citrate solution. Heparinized rabbit plasma was also used: 500 Toronto units were given intravenously and the animals bled 10 minutes after.

Sheep and horses.—730 ml. blood from the jugular vein was received into 270 ml. 2·5 per cent sodium citrate.

Human.—This was kindly supplied by Dr. A. Aubert of the Blood Transfusion Service, Emergency Medical Service, Nottingham, and Lt.-Col. Metcalfe of the Army Blood Transfusion Service, Bristol.

All plasma was centrifuged 3 times at 3000 rev./min. to remove cells, tested for sterility and normally used without Seitz filtration.

Incubation.

This was carried out at 37° C. The " medical flats " were incubated stagnant in air, the flasks either stagnant or gently rocked on a shaker.

Harvesting the vaccine.

After various periods of growth, with liquid cultures, the organisms were centrifuged and the supernatant Seitz filtered. With coagulated plasma cultures the clot was broken up, allowed to contract, centrifuged, and the serum Seitz filtered. Agar cultures (25 ml.) were extracted with 10 ml. saline after breaking up the medium.

Production of Anthrax Oedema Fluid.

This was used as a comparison in assessing the efficacy of culture filtrate vaccines. The method of preparation was similar to that used by Cromartie, Watson, Bloom and Heckly (1946). A suspension of " Vollum " spores containing 10^5 spores in 0·25 ml. TMB was inoculated intracutaneously into the shaved skin of 6 rabbits in 4 or more areas. The animals were killed when about to die. (3–5 days), the oedematous tissue cut out, frozen and coarsely minced. It was then passed through a Latapie mincer, an equal volume of saline being added during the process. The extract was centrifuged clear from gross particles and Seitz filtered.

Animal Tests.

Animals used.

Most of the work has been carried out in rabbits (cross-bred, weight 4·5–5 lb.). Guinea-pigs, mice (albino), sheep (cross-bred Cheviot/Border Leicester) and monkeys (*Macacus rhesus*) were also used.

Active immunization tests.

Route of inoculation.—Intracutaneous, subcutaneous and intravenous routes were used with rabbits. Unless otherwise stated, the route was intracutaneous into the shaved skin of the left flank. Mice, guinea-pigs, sheep and monkeys were inoculated subcutaneously.

Number of doses and time intervals.—The procedure, unless otherwise stated, was that found satisfactory by Cromartie, Watson, Bloom and Heckly (1946), in their studies on oedema fluid. Three weekly doses were used and the animals challenged 1 week after the last dose.

Challenging dose.

A suspension of " Vollum " spores was used. For rabbits the usual challenging dose was 100 A.L.D. and consisted of 10^5 spores in 0·25 ml. TMB given intracutaneously. Guinea-pigs were also given this dose. In spite of their reputed greater susceptibility compared with rabbits, this represented approximately 100 A.L.D. tested under these conditions. Mice were challenged with 10 A.L.D., i.e. 3000 spores in 0·1 ml. given intraperitoneally. Sheep were given 125 A.L.D. (10^5 spores in 0·25 ml. TMB). In all cases animals were injected on the opposite side to that in which the vaccine was given.

Passive immunization tests.

These were carried out on mice and rabbits using sera from hyperimmunized rabbits and sheep. The sera were given intravenously in stated doses. Twenty-four hours after the animals were challenged as with the active immunity tests.

In vitro *Immunological Tests.*

Precipitation.

Hyperimmune sera from rabbits and sheep and commercial anti-anthrax horse serum were tested against serial dilutions of culture filtrates. Readings were made after 1 hour at 37° and overnight in the ice-chest.

Complement fixation.

The technique was similar to that of Fildes and McIntosh (1918). The test carried out with $2\frac{1}{2}$ and 5 M.H.D. complement. Adequate controls were included.

EXPERIMENTAL.

The Use of Anthrax Oedema Fluid as a Culture Medium.

As mentioned above, oedema fluid produced by non-specific agents in rabbits, e.g. turpentine, used as a culture medium gave entirely negative results. Specific oedema fluid was investigated. On the hypothesis that the antigen was produced in the body by the multiplication of the organism locally in the inflammatory fluid, this must contain or have contained the metabolities essential for the production of the antigen. It was considered possible that the fluid was not harvested at the point where these metabolites would be exhausted. To test this, oedema fluid heated at 60° C. for $\frac{1}{2}$ hour to destroy the antigen was inoculated

with 10^6 spores of the "Vollum" strain. The culture grew heavily, and was reaped after 18 hours. Filtrates tested in rabbits were inactive. There remained the possibility that the metabolites had been destroyed by heating, and an attempt was made to discover whether an increased amount of antigen would be found in filtrates of cultures in unheated oedema fluid. Heavy growth was obtained from small inocula. 17-hour culture filtrates tested in rabbits gave an unexpected result. The fluid had completely lost its activity. Destruction by the growth of the organism was also apparent after 7 hours: three doses of 0·5 ml., which protected all of 5 rabbits before culture, only protected 2 out of 5 after. Destruction is due to a soluble substance present in culture filtrates, probably the proteinase, since fluid incubated 3 days at 37° C. with sterile filtrates from 48-hour broth cultures of anthrax had also lost its activity. Dr. H. N. Rydon found that 85 per cent. of the protein in such mixtures had been rendered non-precipitable by protein precipitants. Some loss was also found in fluid incubated for the same time in saline. This may have been due to anthrax proteinase already present in the fluid.

These observations showed that it was necessary to test filtrates at different times during growth in order not to miss antigen which may have been formed and later destroyed.

Filtrates from Rabbit Serum Cultures.

Bloom, Watson, Cromartie and Myer (1946) have shown that the organism contains substances capable of neutralizing the anthracidal substance present in rabbit serum and tissues. In order to obtain growth in rabbit serum, it was necessary to use an inoculum containing sufficient numbers of organisms to produce the necessary concentration of the neutralizing factor. At least 3×10^8 spores in 25 ml. culture were required. Filtrates in undiluted serum and in TMB or CCY broth containing 10–50 per cent serum reaped at various times between 4 and 42 hours were consistently negative when tested in rabbits.

Filtrates from Rabbit Plasma Cultures.

As with serum, owing to the anthracidal substances present, growth could only be obtained in citrated plasma if large inocula were used. It was found, however, that if the plasma were clotted, growth was obtained with an inoculum more than 1,000 times less than that allowing growth in liquid cultures. This was attributed to a "nidus effect," a small collection of organisms localized by the coagulum being able to produce a local concentration of factor sufficient to counteract the anthracidal activity of the plasma. It was, however, necessary to remove cells (leucocytes and platelets) as completely as possible, otherwise growth was poor and in some cases non-existent. Seitz filtration (after the preliminary passage through the filter of 2·5 per cent sodium citrate to remove calcium) was unsatisfactory; it appeared to interfere with subsequent clotting. A glass sintered funnel (No. 4, porosity 5–15μ) was found satisfactory, and the plasma was filtered through this before use.

Cultures were put up as follows: 25 ml. citrated plasma in a "medical flat" was inoculated with 1·0 ml. TMB containing known numbers of spores, mixed, and 5 ml. 2·5 per cent $CaCl_2$ (autoclaved) added. The bottle was laid flat and the plasma allowed to clot. It was incubated stagnant at 37° C. Growth occurred

as small colonies distributed through the medium, but particularly numerous where the clot was in contact with the glass. Only about one-tenth of the number of spores inoculated grew, showing that some anthracidal effect was still apparent. Three types of colonies were found: (1) dense opaque, (2) like fine thistledown, (3) like glucosazone crystals. Each of these colonies gave rise to all three types when transferred to fresh medium. They were not found with sheep, horse or human plasma, where the colonies were all similar to (1). It is possible that they represented various stages in resistance to the bactericidal substances present. Microscopically, organisms from colonies (1) and (3) were well capsulated; (2) appeared to be devoid of capsules. The culture was reaped after various times of growth, the clot being broken up, allowed to contract, and the serum Seitz filtered.

In contradistinction to the results with rabbit serum, these filtrates were highly active. Complete protection in rabbits was obtained with 3 weekly doses of 1·0 ml., and filtrates could be diluted to $\frac{1}{8}$ before the activity became greatly reduced. To avoid repetition, active filtrates will be referred to as PCF prefixed with the name of the animal from which the plasma has been obtained.

Two factors appeared to be of major importance: (1) size of culture inoculum, (2) age of culture.

Size of culture inoculum.

Table I shows the effect of different sized inocula on the production of protective antigen in rabbit plasma cultures (Rabbit PCF).

With the inoculum of 10^4 spores, filtrates were as active as oedema fluid. With an inoculum of 10^8 or greater, little or no antigen was found. This probably explained the negative results of tests of cultures in rabbit citrated plasma or serum where 3×10^8 spores were inoculated. Unless otherwise stated, 10^4 spores per 25 ml. culture were used in all subsequent tests.

Age of the culture.

Table II shows the importance of the time of reaping.

It can be seen that little antigen was formed at 12 hours. At 48 hours it was destroyed. Reapings were normally made at 18 hours.

Plasma from Other Animals.

Guinea-pig, human, sheep and horse plasmas were tested. These did not contain anthracidal substances nor was the presence of cells inhibitory. It was unnecessary to filter such plasma through sintered glass. The first sample of human plasma tested gave very poor results. When evidence as to the importance of pH was obtained (Table VI), it was found that different batches of human plasma as received from the blood depots varied considerably in pH. Some batches were as low as the critical level 6·5. When this was controlled, much more active filtrates were obtained. Table III shows the results with filtrates from different animal plasmas.

All plasmas showed activity. Subsequent work was mainly confined to sheep plasma.

TABLE I.—*The Effect of Different Sized Inocula in Rabbit Plasma Cultures on the Production of PCF. Filtrates Harvested after* 18 *hours and Injected into Groups of* 2 *Rabbits* (3 *Weekly Doses i.c.*). *Challenge* 100 *A.L.D.*

Culture inoculum (viable spores).	Dose vaccine (ml.).*	Number of rabbits surviving/number tested.	
10^4	1·0	2/2	
	0·25	2/2	
	0·125	1/2	(9)†
10^6	1·0	2/2	
	0·25	2/2	
	0·125	0/2	(2, 4)
10^7	1·0	2/2	
	0·25	1/2	(3)
	0·125	0/2	(2, 6)
10^8	1·0	0/2	(5, 7)
	0·25	0/2	(3, 4)
	0·125	0/1	(3) 1 died during immunization.
10^9	1·0	1/2	(3)
	0·25	0/1	1 died during immunization.
	0·125	0/2	(3, 4)
Controls: No vaccine	—	0/4	(4, 4, 4, 5)
Normal rabbit serum	1·0	0/2	(2, 3)
Oedema fluid	0·5	4/4	
	0·25	3/4	(14)
	0·125	2/3	(4) 1 died during immunization.

* In this and all subsequent experiments, the doses of vaccine were made up to 1·0 ml. with saline where necessary before injection.

† The numbers in brackets refer to the time of death in days.

TABLE II.—*The Effect of the Age of the Culture on the Production of Rabbit PCF. Inoculum* 10^4 *Spores.* 3 *weekly doses. Challenge* 100 *A.L.D.*

Age culture at time reaping (hours).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.	
12	1·0	1/1	1 died during immunization.
	0·25	0/2	
	0·125	0/2	
18	1·0	2/2	
	0·25	2/2	
	0·125	1/2	
30	1·0	2/2	
	0·25	2/2	
	0·125	1/2	
48	1·0	0/2	
	0·25	0/2	
	0·125	0/1	1 died during immunization.
Controls: No vaccine	—	0/4	

TABLE III.—*Presence of PCF in Culture Filtrates with Plasma from Different Species. Cultures Reaped at* 18 *hours. Three weekly doses of Vaccine* (*i.c.*) *in Groups of Rabbits. Challenge* 100 *A.L.D.*

Animal from which plasma was obtained.	Culture inoculum (spores).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.
Sheep	10^3	1·0	2/2
	10^4	1·0	2/2
		0·5	2/2
		0·25	2/2
		0·125	1/2
		0·0625	1/2
	10^6	1·0	2/2
	10^9	1·0	3/4
Horse	10^3	1·0	1/2
	10^5	1·0	2/2
	10^6	1·0	0/2
Guinea-pig	10^4	1·0	2/2
Human (pH 7·0)	10^4	1·0	4/4
		0·5	6/6
		0·25	3/4
		0·125	2/6
Controls : No vaccine		—	0/4

Properties of PCF from Sheep Plasma Cultures.

Protective effect in rabbits.

A summary of the results of 13 experiments using 91 rabbits is given below, using the standard three weekly doses i.c. and challenging one week later with 100 A.L.D.

Dose vaccine (ml.).	Number of rabbits surviving/number tested.
1·0	31/31
0·5	15/17
0·25	17/20
0·125	13/18
0·0625	3/5
Controls	0/52

Degree of immunity produced in rabbits.

Animals killed 14 days after challenge with the usual 100 A.L.D. showed no evidence of harbouring the organism in the spleen or other parts of the body. Where the animal had been given sufficient vaccine to produce a solid immunity, no evidence of a local lesion at the site of challenge was found. With less immune animals the presence of a nodule which might become necrotic and ulcerate similarly to that described by Cromartie, Bloom and Watson (1946) was noted. In still less immune animals there was considerable oedema which tracked

ventrally. If the animal survived this did not resolve, but became purulent, and it was quite common to find in these partially immune animals a large cold abscess filled with thick doughy pus which, however, was completely sterile.

To determine whether rabbits would resist greater challenging doses than 100 A.L.D., groups of 2 rabbits were given the usual three weekly doses of 1·0 ml. sheep PCF and challenged with 10^6, 10^7 and 10^8 spores. These represent 1000, 10,000 and 100,000 A.L.D. respectively. All the animals survived. Post-mortem examinations were carried out in two animals challenged with 100,000 A.L.D. and killed 14 days after. From a small area of necrosis in the centre of small, very localized fibrotic lesions at the sites of injection a few organisms were cultured. No organisms were seen microscopically. Growth could be obtained from saline suspension of the necrotic tissue heated at 60° for 1½ hours. The organisms present were, therefore, spores which had not germinated. Livers, spleens, regional lymphatic glands, blood and bone marrow were completely sterile.

Number of injections required for immunity.

Table IV shows that a minimum of two weekly injections were necessary. In order to allow a safety margin, three doses were normally used. Cromartie and Julianelle (personal communications) also found a minimum of two doses necessary with oedema fluid.

TABLE IV.—*Effect of Number of Doses of Sheep PCF on Immunity Response in Rabbits. Challenge* 100 *A.L.D.*

Number weekly doses (i.c.).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.
1*	1·0	0/2
	0·5	0/2
	0·25	0/2
	0·125	0/2
2	1·0	2/2
	0·5	2/2
	0·25	2/2
	0·125	0/2
3	1·0	2/2
	0·5	2/2
	0·25	2/2
	0·125	2/2

* A further test was carried out with a single dose of 1·0 ml. in 4 animals. Two survived.

Route of inoculation.

This was found to be unimportant. No significant difference was found between intracutaneous, subcutaneous and intravenous routes.

Thermolability.

This was of the same order as the antigen in oedema fluid (Watson, Cromartie, Bloom, Kegeles and Heckly, 1946). It was completely destroyed at 60° C. in 1½ hours. Further experiments with shorter times and lower temperatures are being undertaken.

Absence of toxicity.

There was no indication of the presence of an inflammatory factor in PCF such as that described by Cromartie and his colleagues in oedema fluid. Some evidence of local sensitization with the second and third intracutaneous doses was obtained in rabbits, but this was no more marked than a control with normal sheep serum and was attributable to the foreign protein. Homologous PCF gave very slight local erythema after the second and third intracutaneous injections. When given subcutaneously no reaction was observed. Human PCF was injected subcutaneously in man in three weekly doses. No reaction, either general or local, was obtained.

It may be remarked that, in contrast to the results of the American workers, oedema fluid produced in this laboratory using the "Vollum" strain was also devoid of toxic factor.

PCF has not yet been tested for aggressive action. Cromartie (personal communication), however, was unable to confirm Bail's observations on the aggressive action of oedema fluid.

Storage tests.

Samples of sheep PCF were stored at H ion concentrations ranging from pH 4·9–8·76 at room temperature and in the ice-chest. The pH was adjusted by the addition of small quantities of HCl and NaOH. The samples were tested at intervals for their protective effect in rabbits.

When stored at room temperature at pH 7·12 for 180 days, ·3 doses of 0·25 ml. still protected both of two rabbits. On either side of this pH, however, considerable loss in activity was found. At pH 8·76 no significant loss was obtained after 29 days, but complete loss (0·5 ml. doses) in 180 days. At more acid pH values the loss was more rapid. At pH 5·95 loss in activity was apparent after 29 days and was complete in 180 days. At pH 4·9 loss was evident after 9 days.

When stored in the ice-chest (+ 1° C.) no significant fall in activity was found between pH 5·95 and 8·76 in 180 days. Activity, however, at pH 4·9 showed evidence of loss in 29 days and was completely lost in 180 days. Watson, Cromartie, Bloom, Kegeles and Heckly (1946) also noted the instability of oedema fluid antigen at low pH.

Nature of the Antigen.

As the American workers found with their oedema fluid antigen, chemical fractionation of PCF was difficult. Dr. H. N. Rydon showed that it was non-dialysable and probably protein in nature. He attempted to fractionate it by both alcohol and ammonium sulphate precipitation. No clear-cut separation was obtained; moreover, there was some evidence of destruction. It was decided to investigate the possibility of simplifying the medium used in the production of PCF before continuing further attempts at fractionation (see below).

Production of PCF by Strains other than "Vollum."

Table V gives the results of tests in rabbits using sheep plasma culture filtrates of the eight other strains of *B. anthracis*. Four strains of *B. cereus* were also included, none of which, however, was capsulated.

TABLE V.—*Production of PCF by Various Strains of* B. anthracis *and* B. cereus. *18-hour Culture Filtrates in Sheep Plasma Tested in Groups of Rabbits. Three weekly doses of* 1·0 *ml. Challenge* 100 *A.L.D. "Vollum" Spores.*

Strain.	Virulence.	Spores.	Capsules.	Number of rabbits surviving/number tested.	
B. anthracis :					
"Weybridge"	0	+	0	8/8	
"Hagan"	+	+	+	3/3	1 non-specific death.
"M36"	+++	+	+	4/4	
"HM"	0	0	+ (on agar, in air)	0/4	
N.C.T.C. 109	?	+	+	0/4	
1711	0	0	+	4/4	
5444	0	+	+	0/4	
2620	?	+	+	2/4	
"Vollum" (control)	++	+	+	8/8	
B. cereus. All of four strains N.C.T.C. 945, 2599, 2601, 6349				0/4	

The culture inoculum of the non-sporing strains was standardized to an opacity equivalent to 10^5 spores/ml. It is not known what this represents in viable bacilli. Assuming a bacillus has the size of 4 spores and allowing for a difference in viability between spores and bacilli, the inoculum was estimated to be approximately 2×10^4 bacilli.

From Table V it will be seen that there does not appear to be any relation between virulence, sporulation or capsulation and the production of PCF. No reputedly virulent strain, however, was devoid of activity. Of the avirulent strains, two are noted to be effective living vaccines: N.C.T.C. 1711 (Pasteur *premier vaccin*) and "Weybridge." These both produced PCF. Strain N.C.T.C. 5444 which was devoid of activity was tested as a living spore vaccine in rabbits. The doses and time intervals were, as far as possible, the same as those giving full protection with the commercial "Weybridge" vaccine, *viz.*, one dose each of 5×10^7 and $2{\cdot}5 \times 10^8$ spores at 14 days' interval in 8 rabbits. Challenge dose of 100 A.L.D. "Vollum" spores was given 14 days later. All animals died. There appears, therefore, to be a correlation between efficiency as a living vaccine and ability to produce PCF. In this connection it may be noted that oedema fluid produced by avirulent non-capsulated vaccine strains is known to contain the protective antigen (Stamatin and Stamatin, 1936; Grabar and Staub, 1946).

Test of the Presence of PCF in Organisms.

Capsulated organisms grown in broth aerated with air + CO_2.

Cultures of "Vollum" strain in CCY broth were aërated with air containing 10 per cent CO_2 as described above. In 18 hours all organisms were capsulated. They were centrifuged, the supernatant broth removed and the deposit suspended in saline. They were mechanically disintegrated by a modification of the method used by Curran and Evans (1942). Ten ml. suspensions of opacity 40 (Burroughs Wellcome vaccine opacity standard) were placed in 2-oz. screw-capped bottles containing 10 g. glass beads (Ballotini No. 13, 0·228 mm. diam.). 0·1 ml. tributyl

citrate* (anti-frothing agent) was added, the screw caps replaced, and the whole shaken for 2 hours vertically at 400 shakes/min. (amplitude 2 in.). Microscopically, the appearance was an amorphous mass staining pink and blue with methylene blue in which no organism was seen. Plating showed that the suspensions had been sterilized. Three weekly injections of 1·0 ml. subcutaneously of $\frac{1}{4}$ dilution gave no protection in 4 rabbits when challenged one week later with 100 A.L.D. "Vollum" spores. Eight other rabbits were injected with doses ranging from 0·3 ml. of a dilution $\frac{1}{4}$ to 1·2 ml. given intravenously three times a week for 6 weeks. Two were challenged 2 months after the last dose. Both succumbed. The organisms grown under these conditions do not, therefore, appear to contain the protective antigen.

Organisms grown in plasma cultures under conditions giving optimum PCF production in the culture filtrate.

The bacilli from ten 18-hour cultures in 25 ml. sheep plasma were suspended in saline, freed from plasma clot by filtration through muslin and washed twice with 200 ml. saline. They were then suspended in 10·5 ml. saline and the opacity of the suspension determined (opacity 32, Burroughs Wellcome standard). It was diluted 1/3·2 placed in 2-oz. screw-capped bottles, tributyl citrate (0·1 ml.) added and shaken for 2 hours as described above. A control was used consisting of 10 ml. PCF *plus* 0·1 ml. tributyl citrate shaken under the same conditions. The suspension was tested for sterility, and groups of 4 rabbits were given three weekly injections i.c. of 1·0 ml. of each material and challenged in the usual way. With the organisms, all four animals died, in spite of the fact that the dose in each rabbit corresponded to the number of organisms present in 22·5 ml. culture, or about 30 times the amount of filtrate sufficient to give complete protection (3 doses of 0·25 ml.). With PCF shaken for the same time under the same conditions, 3/4 survived. Some destruction of PCF due to shaking was therefore apparent, but it is unlikely that the complete absence of activity in the organisms could be accounted for in this way. It seems, therefore, that the antigen is produced extracellularly, and is not present in the organisms even under conditions where active antigen is produced.†

Protective Tests in Animals other than the Rabbit.

Guinea-pigs.

Rabbit, guinea-pig and sheep plasma culture filtrates were used in groups of 6–8 guinea-pigs in doses of 1·0 ml. injected subcutaneously once a week for three weeks. The challenge was 100 A.L.D. spores. Of 12 injected with sheep PCF, only 1 survived 14 days. There was definite evidence of prolonged survival time in the others, all of 16 controls being dead within 4 days, and 5 injected animals surviving 5–11 days. The guinea-pig PCF also gave prolonged survival time in 7 animals used (mean 5·4 days) compared with the controls (mean 3·5 days). No evidence of protection was found with rabbit PCF.

These results are in agreement with most other workers, who find that the

* This was shown to have no action on the antigen.

† An alternative suggestion has been communicated by Mlle. Staub that the antigen was destroyed by the proteinase liberated from the ruptured cells. This hypothesis is being tested by further experiment.

guinea-pig is much more difficult to immunize than the rabbit. White's results (1946*b*) suggested that, unlike the rabbit, in the guinea-pig the immunity is not sufficient to destroy the spores, and they may remain latent in the body of the guinea-pig and may germinate to give late deaths. If this is so, it might be possible to produce complete protection against a vegetative inoculum, although failing to do so with spores.

Mice.

Groups of 10 mice were injected with 0·5–1·0 ml. sheep and human PCF subcutaneously and intravenously. Three and four weekly injections were made and the mice challenged with 10 A.L.D. spores intraperitoneally. No evidence of any protection was obtained.

Sheep.

Three doses of 10 ml. sheep PCF given subcutaneously protected all of three sheep against 125 A.L.D. spores injected one week later. Three controls died in 3 and 4 days. These sheep were subsequently hyperimmunized to produce sera. One sheep which had received a further three doses of 10 ml. sheep PCF was injected with a suspension of living capsulated " Vollum " strain from an 18-hour culture, the bacterial substance being equivalent in opacity to 10^8 spores. Assuming the vegetative organism was four times the size of a spore, this would represent about 30,000 A.L.D. It survived, no reaction, general or local, being observed.

A further test with 2·0 ml. doses was carried out by Lt.-Col. J. Barnes in two sheep. Three weeks after challenge with 125 A.L.D. both animals were alive and well.

Monkeys.

Groups of 2 *Macacus rhesus* were given three weekly doses subcutaneously of 1·0 ml. sheep and human plasma culture filtrates. One week later they were challenged subcutaneously with 10^7 spores (approximately 100 A.L.D.). The controls died in 4 and 5 days. All the vaccinated monkeys survived, and no reactions were observed.

Analysis of the Culture Medium to Determine the Constituents Responsible for the Production of PCF.

Thermostability of the essential constituent.

A series of 18-hour cultures was put up with sheep plasma heated to different temperatures before inoculation. Cultures heated 1½ hours at 56° C. grew well, but clotting was inhibited. All of 7 rabbits injected with the usual three doses of 1·0 ml. survived 100 A.L.D. With plasma heated 1½ hours at 70° C., 1 out of 6 survived. The plasma was coagulated so that it was difficult to estimate the amount of growth, but films showed that growth was appreciable. Plasma steamed and autoclaved 20 min. 115° C. was completely ineffective. It would seem therefore that the essential constituent was destroyed at 70° C. for 1½ hours, the coagulation temperature of proteins.

The effect of pH on the production of PCF.

Table VI shows the effect of growth at different pH on the production of PCF in sheep plasma cultures.

TABLE VI.—*The Effect of pH on Production of PCF. Protective Effect in Rabbits Tested under the Standard Conditions. Culture: Sheep Plasma, Inoculum* 10^4 *Spores; 18 hours' growth.*

Initial pH.	Approximate estimation of amount growth (no. colonies × 10^3).	Final pH.	Dose PCF i.c. (ml.).	Number of rabbits surviving/ number tested.
8·43	2	7·6	0·5	2/2
			0·25	1/2
			0·125	1/2
			0·0625	1/2
7·66	5	7·6	0·5	1/2
			0·25	2/2
			0·125	2/2
			0·0625	1/2
6·96	10	7·0	0·5	2/2
			0·25	2/2
			0·125	1/2
			0·0625	1/2
6·5	10	6·5	0·5	0/2
			0·25	0/2
			0·125	0/2
			0·0625	0/2
6·04	5	6·2	0·5	0/2
			0·25	0/2
			0·125	0/2
			0·0625	0/2
5·6	No clot, poor growth	6·2	0·5	0/2
			0·25	0/2
			0·125	0/2
			0·0625	0/2

Table VI shows a very clear-cut result: PCF was not produced in cultures at pH 6·5 or lower. There is no significant difference between results with filtrates produced at pH 6·96–8·43. This is not due to a direct effect of pH on PCF, since it can be stored overnight at 37° C. at pH 6·0 without loss.

Fractionation of Plasma to Determine the Metabolites essential for the production of PCF.

Serum.—The experiments recorded above were all carried out using whole plasma. Tests were now made with serum.

Rabbit serum.—It has been pointed out that cultures in rabbit serum failed to grow unless large inocula were used, and that with such inocula no PCF was obtained. Growth, however, could be produced with small inocula (10^4 spores)

if the medium were made solid or semi-solid with agar (1·0–0·5 per cent). Saline extracts of such cultures (25 ml. extracted with 10 ml. saline) protected 2 of 4 rabbits tested with doses equivalent to 1 ml. undiluted serum. Heparinized plasma similarly treated gave no better protection, so that the results cannot be attributed to a difference between serum and plasma, but rather to the interference by agar of PCF production.

Sheep serum.—There are no substances inhibitory to growth in sheep serum, and undiluted serum cultures inoculated with 10^4 spores grew well. Filtrates of 18-hour cultures tested in falling doses in rabbits under the standard conditions gave the following results :

Dose vaccine (ml.).	Number of rabbits surviving/number tested.
1·0	9/10
0·5	3/5
0·25	4/5
0·125	3/5
0·0625	1/5
Control (no vaccine)	0/5

If these results are compared with the results obtained with plasma there is a slight indication that sheep serum is less effective than plasma ($\chi^2 = 6{\cdot}15$; $P = 0{\cdot}047$). For the comparison, since the doses are the same in each series, a total of all animals surviving irrespective of dose has been taken. Further experiments, however, comparing dilutions of plasma and serum in culture media, showed that there was little difference between them (Table VII) ($\chi^2 = 0{\cdot}095$).

TABLE VII.—*A Comparison between the Protective Effects in Rabbits of Filtrates of Cultures in Various Dilutions of Sheep Plasma and Serum. Standard 3 weekly doses 1·0 ml. i.c. Challenge 100 A.L.D.*

Dilutions of plasma* or serum in culture.	Number of rabbits surviving/number injected with serum culture filtrate.	Number of rabbits surviving/number injected with plasma culture filtrate.
Undiluted	9/10	4/4
1/2	3/4	4/4
1/4	5/6	5/6
1/8	3/8	5/7 (1)
1/16	3/7 (1)†	4/8
1/32	1/3 (1)	0/4
Control (filtrate from culture in 25 per cent TMB)	0/4	
Control (no vaccine)	0/4	

* In order to keep the amount of growth as constant as possible TMB was added in a final concentration of 25 per cent. It has been found that this medium gave approximately the same amount of growth in 18 hours as undiluted sheep serum. With plasma cultures, $CaCl_2$ was added in amounts sufficient to remove the citrate ion. No clotting, however, occurred at dilution of 1/8 and lower.

† Numbers in brackets refer to non-specific deaths during immunization.

The effect of dialysing serum and plasma.

Initial experiments were done with sheep plasma. Later, serum was used with similar results. With plasma the dialysis, carried out by Dr. Rydon, was as follows :

250 ml. citrated plasma dialysed against 750 ml. distilled water for 17 hours

Residue.	*Diffusate.*
Dialysed against 0·67 per cent sodium citrate in 0·9 per cent saline for 2 days with frequent changes. Volume 270 ml.	Concentrated *in vacuo* below 35° C. to 120 ml. (contains 75 per cent of diffusible material).

Serum was dialysed in the same way except that citrate was not used in the saline. Each fraction was Seitz filtered and cultures were put up as follows, using as a control Seitz filtered plasma. In order to keep the amount of growth constant, TMB was added to a concentration of 25 per cent.

(1)	Diffusate 16 ml.				+	TMB 6 ml.	+	saline 3·2 ml.				
(2)	„ 8 „				+	„	+	„ 11·2 „				
(3)			Residue 16 ml.		+	„			+	2·5% $CaCl_2$	3·2 ml.	
(4)			„ 8 „		+	„	+	„ 9·6 „	+	„	1·6 „	
(5)	Diffusate 8 „	+	„ 8 „		+	„	+	„ 1·6 „	+	„	1·6 „	
(6)	Plasma 16 „				+	„			+	„	3·2 „	
(7)	„ 8 „				+	„	+	9·6 „	+	„	1·6 „	

All cultures remained fluid, clotting in (6) and (7) being inhibited by Seitz filtration. In 18 hours little difference was noted in the amount of growth, but growth in (3) and (4) was granular. Capsule production was well marked in cultures (5), (6) and (7), but was absent in cultures (1), (2), (3) and (4). 18-hour culture filtrates were tested in rabbits using the standard conditions (Table VIII).

TABLE VIII.—*Evidence of Two Factors in Plasma Required for the Production of the Immunizing Antigen. Groups of 4 Rabbits Immunized under the Standard Conditions with Culture Filtrates in Fractions of Plasma Separated by Dialysis.*

Culture filtrate (see text).	Approx. concentration (per cent of plasma).	Dose vaccine (ml.).	Number of rabbits surviving/number tested.
(1) Diffusate	100	1·0	0/4
(2) „	50	1·0	0/4
(3) Residue	57	1·0	0/4
(4) „	28·5	1·0	0/4
(5) „ + diffusate	50 + 28·5	2·0	4/4
(6) Whole plasma	60	1·0	4/4
(7) „ „	30	1·0	4/4

A similar result was obtained with dialysed serum.

It is clear from this result that two factors present in plasma are both necessary. One of these factors is dialysable, the other is retained. That the two factors are required in the production of the immunizing antigen and not in its protective effect is shown by pooling culture filtrates (1) and (3) (Table VIII) and testing the mixture in rabbits. No protection was obtained.

Estimation of the amount of each factor required.

Cultures were put up containing a constant amount of one factor and falling concentrations of the other. Table IX shows the results when filtrates from these cultures were tested in rabbits.

TABLE IX.—*The Optimal Concentrations of each of the Two Factors in Plasma Cultures Necessary for the Production of the Immunizing Antigen. Tests in Rabbits :* 1·0 *ml. Filtrate given i.c.* 3 *weekly doses. Challenge* 100 *A.L.D.*

Basal medium : *TMB diluted to* 25 *per cent after addition of factors. Final volume* 25·7 *ml. Inoculum* 10^4 *spores in* 0·5 *ml. TMB.* 2·5 *per cent* $CaCl_2$ *was added in the proportion of* 1·6 *ml. to* 8 *ml. dialysed plasma to remove the citrate ion.*

Diffusate } *As in Table VIII.*
Residue }

Residue.		Diffusate.		Per cent organisms showing capsules.	Number of rabbits surviving/number tested.
Volume (ml.	Per cent whole plasma.	Volume (ml.).	Per cent whole plasma.		
8	29	8	50	75	4/4
8	29	2	12·5	50	3/4
8	29	0·5	3·1	v. few	1/4
8	29	0·125	0·8	0	1/4
8	29	0	0	0	0/4
4	14·5	8	50	75	4/4
2	7·2	8	50	75	3/4
1	3·6	8	50	75	2/3 (1)
0·5	1·8	8	50	50	2/4
0	0	8	50	0	0/3 (1)
Control (no vaccine)					0/4

Similar results were obtained with serum.

Numbers in brackets refer to non-specific deaths during immunization which have been excluded from the denominator.

From these results it would seem that dialysed plasma cannot be diluted below 14·5 per cent and diffusate not below 50 per cent without loss of activity. It would also appear that under these conditions both factors are required also for capsule production. However, there is no question of capsulation playing a part, since the " Weybridge " strain produced no capsules, and yet in the presence of both factors gave active culture filtrates.

Replacement of the diffusate factor with sodium bicarbonate.

It was found that the diffusate factor could be replaced by sodium bicarbonate. A solution in saline (*M*/2 ; 4·2 per cent) was sterilized by filtration and added to cultures containing 29 per cent dialysed sheep plasma and 25 per cent TMB to make concentrations of 1·0, 0·2 (approximately equivalent to the concentration in plasma) and 0·04 per cent. Cultures were incubated for 18 hours after inoculation with 10^4 " Vollum " spores. With 1·0 per cent, some inhibition of growth was found, presumably due to the high pH.

Capsulation was well marked with 1·0 and 0·2 per cent, but was absent with 0·04 per cent., showing that the factor responsible for capsulation in the diffusate

could also be replaced by bicarbonate. Tests in groups of 3 rabbits under the standard conditions gave complete protection with 1·0 and 0·2 per cent. With 0·04 per cent, all 3 died. A concentration of bicarbonate equal to that in plasma, therefore, is effective, but whether the activity of the diffusate is entirely dependent on its content of bicarbonate is still under investigation. Table IX suggests that the diffusate factor is active in higher dilutions: 12·5 per cent (equivalent to 0·025 per cent sodium bicarbonate) produced filtrates which protected 3/4 rabbits, whilst 0·04 per cent sodium bicarbonate gave no protection when tested in 3 rabbits. However, with such small numbers of animals it is impossible to obtain anything like an accurate estimate. The effect of sodium bicarbonate was shown not to be due to pH.

Carbon dioxide.

In order to test whether carbon dioxide would replace the dialysate factor, cultures in 29 per cent dialysed plasma and 25 per cent TMB were incubated in equilibrium with 5 per cent CO_2 in air. The cultures were saturated with air containing this concentration of CO_2 and incubated stagnant in McIntosh and Fildes' jar filled with the gas. The pH fell to 6·5 immediately after gassing. A good capsulated growth was obtained, but filtrates were inactive. The concentration of HCO_3' in equilibrium with 5 per cent CO_2 at pH 6·5 is 0·00285 *M*. This corresponds to a concentration of $NaHCO_3$ well below that found to be effective in producing the antigen. At higher pH values the equilibrium would shift so that more and more bicarbonate would be present. It is impossible therefore to test the effect of CO_2 in the virtual absence of bicarbonate ions at pH values above the critical value for the production of the antigen, i.e. pH 6·5 (Table VI). The relation between CO_2 and capsule production and the use of CO_2 as a standard method of producing capsulated organisms has been pointed out. It seems, therefore, that for capsule production, in contrast to the production of the immunizing antigen, CO_2 and bicarbonate are interchangeable. However, it is possible that the effect of bicarbonate depends on the CO_2 with which it is in equilibrium.

The nature of the residue factor retained on dialysis.

Little work has so far been done. Preliminary tests on dialysed serum fractionated by Dr. Rydon indicated that it was associated with the albumen fraction. Further work is in progress.

Passive Immunization Tests.

Preliminary tests were carried out with sera from rabbits immunized with 3 weekly doses of 1·0 ml. of rabbit and sheep PCF. The animals were bled from the jugular vein 14 days after the last injection. As a routine the sera were heated ½ hour at 56° C., but unheated sera were also used. The sera were given intravenously as described under "Technical Details" to groups of 10 mice in doses of 0·5 to 0·05 ml. and into groups of 2 rabbits in doses of 10–1·0 ml., and the animals were challenged with 10 A.L.D. (for mice) and 100 A.L.D. (for rabbits) of spores 24 hours after. No evidence of protection in either animal was obtained, although the animals from which the sera were taken had a solid immunity.

Further tests were carried out with sera from hyperimmunized rabbits and sheep. For the preparation of the sera 5 sheep and 12 rabbits were used. They

were given 2 courses of immunization, using 1–10 ml. doses of PCF in sheep and 0·3–4·0 ml. doses in rabbits. Both homologous and heterologous PCF was used in both species. Doses were given either subcutaneously or intravenously at times ranging from 1–3 times per week with 1 week's interval between courses. Bleedings were made between courses, and after the second course 1 week after the last injection. The sera were tested in groups of 20 mice in doses of 0·05–0·5 ml. intravenously and challenged with 10 A.L.D. spores 24 hours later. No indication of protection was obtained. A total of 68 rabbits given doses ranging from 10–50 ml. of these sera intravenously were challenged with 100 A.L.D. spores. There was no protection or even significant delay in death time. In other cases rabbits received two doses of serum, one given 24 hours before and the other 24 hours after challenge, again with negative results. It would seem, therefore, that the sera had no protective value, although the animals from which the serum was taken were solidly immune.

The possibility arose that whole blood might be protective, and heparinized blood from rabbits hyperimmunized with sheep PCF was tested in rabbits in doses of 50, 20 and 10 ml. No protective effect was obtained. That the rabbits were capable of being passively protected was shown by the complete protection obtained with 5 and 10 ml. doses of " Weybridge " commercial serum. This is serum from horses immunized first with the " Weybridge " living spore vaccine, followed by a course of immunization with a virulent strain. In order to determine whether an effective serum could be produced by using a virulent strain, three sheep previously hyperimmunized with sheep PCF were given living capsulated " Vollum " vegetative organisms in three weekly doses of 1 ml. of a suspension corresponding in opacity to 3×10^8, 6×10^8 and $1{\cdot}2 \times 10^9$ spores. After 1 week they were bled and the serum pooled and tested for passive protection in rabbits (Table X).

TABLE X.—*Passive Protection Tests in Rabbits using Pooled Sera from 3 Sheep Immunized with Sheep PCF Before and After a Further Course of Immunization with Living Capsulated " Vollum " Bacilli. Serum given Intravenously. Challenge* 100 *A.L.D.*

Serum from animals given.	Dose serum (ml.).	Number of rabbits surviving/number injected.
(*a*) Two courses immunization with sheep PCF	10	0/4 (3, 3, 4, 6)*
	5	0/4 (3, 3, 4, 5)
	2	0/4 (3, 3, 4, 4)
	1	0/4 (3, 4, 4, 4)
(*b*) Ditto + one course with living virulent bacilli	10	3/4 (6)
	5	2/4 (5, 6)
	2	1/4 (3, 5, 5)
	1	0/4 (3, 3, 4, 5)

* Numbers in brackets = survival time in days of animals which succumbed.

From Table X it is seen that immunization with living " Vollum " organisms produced sera which contained protective antibodies for the rabbit. A similar result was found with serum from rabbits treated in the same way with living " Vollum " organisms following immunization with sheep PCF. As a control, a vaccine was prepared using a suspension of capsulated organisms of the same density broken up with glass beads until all the organisms were killed, as pre-

viously described. The serum from rabbits using this vaccine (following immunization with PCF) gave no protection in rabbits. It would seem, therefore, that the living organism is necessary for the production of protective antibodies, although whether it depends on the virulence of the organism has not yet been determined. There is evidence that it does, since we have failed to obtain protective antibodies in rabbits immunized with the " Weybridge " spore vaccine alone. It may be noted that the serum described by Watson, Cromartie, Bloom, Kegeles and Heckly (1946) as protecting 50–80 per cent of their guinea-pigs was from rabbits not only immunized with oedema fluid antigen, but which had also been challenged twice with large doses of virulent spores. Matsumoto (1924) obtained poor results with filtered oedema fluid, but further immunization with unfiltered fluid containing living organisms was much more successful.

Passive protection in mice with sera from sheep and rabbits immunized with PCF, followed by living capsulated suspensions of " Vollum " strain.

Groups of 10 mice were injected i.v. with 0·5 and 0·2 ml. of serum from rabbits given two courses of immunization with sheep PCF followed by one with living capsulated " Vollum " strain. They were challenged 24 hours after with 10 A.L.D. spores i.p. The difference in numbers of deaths between groups given this serum and other groups given normal rabbit serum was not significant ($\chi^2 = 3{\cdot}3 : P = 0{\cdot}2$). A similar result was obtained with the " Weybridge " anti-anthrax serum, which, as stated above, gave complete protection in rabbits. Tomcsik and Iványovics (1938*b*) showed that sera containing capsular antibody had some protective value in mice, but not in rabbits. It would seem, therefore, that sera from rabbits immunized with PCF + capsulated organisms should be protective in mice in consequence of the capsular antibody they presumably contained. Precipitation tests for capsular antibody, however, using a purified polypeptide preparation (Hanby and Rydon, 1946) were consistently negative, even when sera were taken after two further courses of immunization with capsulated organisms. It appears that the capsule on the organism is a poor antigen. We have had repeated failures in rabbits and sheep not only with this strain, but also with the living " HM " strain. Tomcsik and Iványovics (1938*a*) and Iványovics (1940), although they had more success, found that rabbits respond irregularly, and that differences existed between bacterial strains.

Attempts to absorb the protective antibody in immune sera.

Grabar and Staub (1942) showed that the protective antibody for guinea-pigs was in the pseudoglobulin fraction of immune serum, which, however, gave no precipitation with the capsular polypeptide or somatic polysaccharide. Antibodies against these were in the non-protective euglobulin fraction. Absorption with capsular polypeptide did not remove the protective antibodies for guinea-pigs and rabbits (Staub and Grabar, 1943). Matsumoto (1924) failed to remove the protective antibody from immune sera with killed bacilli even when " animalized " organisms were used from peritoneal exudate or when the bacilli were actually grown in the immune serum. He claimed, however, to have succeeded with filtered oedema fluid. A small precipitate was formed, but it appeared immaterial whether this was filtered off or not. On the other hand, Watson, Cromartie, Bloom, Kegeles and Heckly (1946) completely failed to absorb protective antibody with oedema fluid.

Absorption with PCF.

Two sera were used: (1) " Weybridge " commercial anti-anthrax serum, and (2) pooled sera from sheep immunized with sheep PCF followed by living "Vollum" organisms. Absorptions were carried out with sheep PCF. Equal volumes of undiluted sera and PCF, undiluted and in dilution of 1/2, 1/4 and 1/10, were mixed and allowed to stand 2 hours at room temperature and then overnight in the ice-chest. A higher temperature was avoided to prevent possible destruction of PCF by anthrax proteinase that it might have contained. A slight turbidity was noticed in the tube with the highest concentration of PCF, but no precipitate. It was therefore not centrifuged. A second absorption (or neutralization) was carried out, using a volume of PCF undiluted and diluted as before in quantities equal to the volume of the first absorption mixture, thus diluting the serum 1/4. After placing in the ice-chest overnight, the mixture was injected intravenously into groups of 6 rabbits in doses of 40 ml., equivalent to 10 ml. of unabsorbed serum. A control with unabsorbed serum diluted to an equal amount with saline but otherwise treated exactly like the absorbed sera was included. The animals were challenged with 100 A.L.D. spores 24 hours after. Using the " Weybridge " commercial serum, absorption with undiluted PCF, and PCF diluted 1/2, 1/4 and 1/10 gave survival ratios of 2/6, 5/6, 4/6, 4/6 respectively. The unabsorbed serum, however, only protected 2/6. With the sheep serum, the results with the absorbed serum were 4/6, 2/6, 2/6, 1/6, and with the unabsorbed serum, 1/6. The poor results with the controls is not readily explainable, since this dose of these sera gave 75–100 per cent survival in previous tests. In spite of the somewhat irregular results, however, there is no indication of any absorption or neutralization of the immune serum by PCF.

Absorption with living organisms.

In order to determine whether the protective property of sera was dependent on antibody to an antigen in the living organism, absorption tests were carried out with washed living suspensions of capsulated bacilli and spores of " Vollum " strain. " Weybridge " commercial anti-anthrax serum was used. The absorptions were carried out as follows:

First absorption.

(1) 50 ml. serum + 50 ml. saline.
(2) ,, + 50 ml. spore suspension (3×10^9/ml.).
(3) ,, + 50 ml. capsulated bacilli suspension (opacity equivalent to 3×10^9 /ml. spores).

These were allowed to stand in the ice-chest overnight, and then spun down and the supernatant used for a second absorption.

Second absorption.

(1) Supernatant from (1): 90 ml. + 8 ml. saline (final dilution 1/2·2).
(2) ,, (2): 90 ml. + 8 ml. spore suspension ($1{\cdot}8 \times 10^{10}$) (final dilution 1/2·2).
(3) ,, (3): 60 ml. + 40 ml. capsulated bacilli suspension (opacity equivalent to 3×10^9/ml. spores) (final dilution 1/3·3).

After standing overnight in the ice-chest the suspensions were spun down and the supernatants Seitz filtered and the equivalent of 10 ml. of undiluted serum (i.e. 22 ml. of (1) and (2) and 33 ml. of (3)) injected into groups of 2 rabbits intravenously. 24 hours after the animals were challenged with 100 A.L.D. spores intracutaneously. In groups injected with sera (1) and (3), no deaths occurred. In animals injected with Group (2) serum, 1 died out of 2 injected. This animal died on the 8th day. Controls given normal serum died in 3–4 days.

It would seem, therefore, that no significant absorption by spores or capsulated organisms had taken place.

A further absorption test is being carried out with PCF together with living bacilli and with non-capsulated bacilli.

In vitro *Immunity Tests.*

Sera from animals hyperimmunized with PCF and with PCF and living organisms and other sera containing high titres of antibody against the capsular polypeptide and a polysaccharide isolated from the organism by Dr. H. N. Rydon (personal communication) were tested against sheep and rabbit PCF. Except where a reaction was due to heterologous protein, e.g. with sheep serum immune to rabbit PCF and tested against rabbit PCF, no precipitation was obtained in dilutions of antigen 1/2–1/1024 and 1/2 serum.

Complement-fixation tests were also negative. Owing to the anti-complementary effect of protein present in PCF, tests were controlled with normal sheep and rabbit serum. Specific complement fixation was only obtained where heterologous PCF was used as the immunizing and test antigen. The normal serum control showed that this was due to the heterologous protein.

DISCUSSION.

It would seem from the evidence in this paper that the protective antigen formed in plasma culture filtrates has very similar properties, and is probably identical with that described by the American workers in anthrax oedema fluid, but unlike their material, it is not associated with any inflammatory factor. It is not, therefore, necessary to postulate any interaction between the organism and its host to explain the formation of the antigen. Neither is it necessary to regard its formation as being due to an interaction between anthracidal substances in serum or plasma and substances produced by the organism, since the antigen is formed in plasma which is devoid of such substances. On the other hand, it does not seem to be present preformed in the organism, even under conditions of good production in the medium. This suggests that it is distinct from the antigens of the cell. It is formed early in growth, and is destroyed by the proteolytic enzymes of the organism. It would appear to be, therefore, formed externally by the cell and not a product of autolysis.

Of the two factors in plasma or serum necessary for production of the antigen, one is a serum protein or associated with it, probably albumen. It is possible that the antigen is formed from normal serum albumen by coupling of an active haptene (for example the polysaccharide *a* of Grabar and Staub, 1944) produced by the organism. Preliminary experiments, which will be reported in a subsequent paper, made it clear that the non-dialysable (albumen) factor had to be present in the culture medium. If suspended in the

medium in a cellophane bag, no antigen was formed either inside or outside the bag. Attempts to produce the antigen by mixing normal serum with filtrates from broth cultures containing 0·2 per cent sodium bicarbonate were unsuccessful.

What role the second (dialysable) factor plays it is impossible at this stage to conjecture, although it has been established that it can be replaced by sodium bicarbonate. It does not act as a buffer or by altering the pH of the medium. Some evidence as to whether it is incorporated in the antigen could be sought by the use of bicarbonate containing isotopic carbon. The carbonate or bicarbonate ion appears to be necessary, since it is not replaceable by CO_2.

The apparent absence of protective antibodies in the serum of animals hyperimmunized with this antigen is in accordance with the findings of most workers using oedema fluid or spore vaccines. It raises once again the long-debated question as to whether a true cellular immunity may be produced apart from circulating antibody (Teale, 1935). It is not proposed here to enter into a discussion on this point, except to state that no protective antibodies could be demonstrated in the spleen, liver and bone marrow of immune rabbits ground up in their serum. Neither was whole blood effective.

A further mystery is the nature of the protective antibodies that are produced on further immunization with virulent organisms. The complete failure to obtain any *in vitro* tests, and the failure to absorb or neutralize the antibodies with active culture filtrates or by living organisms, suggests that a still further antigen is involved which is produced by virulent cells only in the body. Yet Watson, Cromartie, Bloom, Kegeles and Heckly (1946) were not able to absorb protective antibody with oedema fluid produced by virulent strains. If such an antigen exists, therefore, it must be so labile that it is destroyed in the extraction process. Another possibility is that, since it is not possible to remove the culture filtrate antigen after absorption, failure to obtain absorption might be due to the formation of a soluble antigen-antibody complex easily dissociated in the animal body. Evidence for a soluble antigen-antibody complex between oedema fluid and the pseudoglobulin fraction of immune serum has been put forward by Grabar and Staub (1944). It is, then, still required to explain why living organisms are necessary to produce circulating antibody against the filtrate antigen. Obviously, considerably more work must be done before these problems can be solved.

Further work on the nature of the protective antigen is also being carried out.

SUMMARY.

1. An antigen having similar properties to that present in anthrax oedema fluid capable of protecting rabbits, sheep and monkeys against 100 or more lethal doses of spores has been produced in filtrates of cultures of *B. anthracis* in plasma or serum of various animals.

2. The size of culture inoculum, the age and pH of the culture are important factors. Destruction of the antigen by the growing organism may occur. No antigen is produced at pH 6·5 or less.

3. Plasma heated to 56° C. for 1/2 hour is still capable of producing the antigen, but not when heated to 70° C.

4. Two factors in plasma or serum are necessary. One is dialysable and can be replaced by sodium bicarbonate. The other is retained and is associated with the serum proteins.

5. The production of the antigen has no relation to virulence, capsulation or sporulation. No virulent strain failed to produce antigen. The production of antigen by avirulent strains was related to their efficacy as living vaccines. No antigen was produced by *B. cereus*.

6. Sera from rabbits and sheep hyperimmunized with the antigen contained no protective antibodies. Further immunization with living virulent bacilli produced effective protective sera. The protective antibodies were not absorbed with the antigen from culture filtrates or with living bacilli.

7. No precipitation or complement-fixation reactions were obtained with hyperimmune sera.

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ADAPTATION OF "NITRATASE" IN WASHED SUSPENSIONS OF BACTERIA.

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IT is now fairly well established that certain microbial enzymes can, under suitable conditions, in the presence of their specific substrate develop increased activity without cell multiplication. This was first reported by Dienert (1900) for yeast galactozymase, and later confirmed by Stephenson and Yudkin (1936), and Spiegelman, Lindegren and Hedgecock (1944). It has also been reported for formic hydrogenlyase of *Bact. coli* (Stephenson and Stickland, 1932), galactozymase of *Str. lactis* (Hegarty, 1939), an enzyme oxidizing *p*-aminobenzoic acid in a soil bacillus (Mirick, 1943), and for tetrathionase of *Salm. paratyphi B* (Knox and Pollock, 1944).

Quastel, Stephenson and Whetham (1925) showed that washed suspensions of *Bact. coli* could reduce nitrate to nitrite in the presence of a suitable H-donator, and the reaction was studied in greater detail by Stickland (1931).

The similarity between nitrate reduction and tetrathionate reduction by intestinal organisms has previously been pointed out (Pollock and Knox, 1943), and it was decided to attempt to discover whether bacterial nitratase would adapt itself in washed suspensions, without cell proliferation, in a manner similar to tetrathionase.

The results of the investigation are reported in this paper; and it will be shown that there is, with respect at least to the organisms most extensively studied, a close analogy between adaptation to nitrate and to tetrathionate. The two processes, though specific and apparently independent, are indeed similar in many respects.

METHODS.

Organism.—The organism used in most experiments was a non-pathogenic Gram-negative coliform bacillus, isolated from human faeces and labelled "1433." It grows exceptionally well on all laboratory media, including a simple ammonia-glucose-salt mixture. It produces acid and gas in lactose, glucose, and mannitol, but not in sucrose or salicin. It is methyl red positive, Voges-Proskauer negative and citrate positive. It produces H_2S but no indole. It falls therefore into the coliform group "Intermediate, type I" (Min. of Health, 1939). It was chosen primarily because, although closely related to *Bact. coli*, it was a powerful reducer of tetrathionate as well as nitrate, and its tetrathionase adapted itself rapidly in washed suspensions, similarly to that of *Salm. paratyphi B*.

A strain of *Bact. coli* and one of *Salm. paratyphi B*, both isolated from human faeces, were used in a few experiments.

Medium.—The stock growth medium was a tryptic digest of meat in 2 per cent agar, distributed in 100 ml. lots in Roux flasks.

Preparation of suspensions.—2 ml. of a saline suspension of organisms grown on an agar slope at 37° for 18 hours were inoculated on to each Roux flask. The flasks were incubated at 37° for 16 hours and the cells washed off with distilled water. The organisms were centrifuged down and further washed with distilled water, being finally made up into a thick suspension in water. 6 to 10 ml. of suspension containing about 2·5 mg. N and 15×10^{10} living organisms per ml. were obtained from each Roux flask. For most adaptation experiments the cells were used at a final dilution of

1 : 10 from this strength. After adaptation, and in order to test for adapted nitratase activity, the cells were further diluted 1 : 10 or 1 : 20—i.e., were used at a final concentration of about 10×10^8 per ml.

Method of following nitrate reduction.—The reaction was followed in evacuated Thünberg tubes, placed in a thermostatically controlled water bath. 0·5 ml. of bacterial suspension was placed in the stopper, and added when required to a mixture containing M/50 KNO_3, M/5 phosphate buffer, pH 7·2, and M/50 H-donator. 1·0 or 0·5 ml. samples were removed at 20 to 30 min. intervals for nitrite estimation; the tube was re-evacuated and replaced in the bath as soon as possible. Even when dealing with 4 tubes at a time (the maximum) the whole procedure of sampling did not take longer than 4 minutes.

Estimation of nitrite.—1 ml. samples of the suspension-mixture (diluted in water, if necessary) were added to 4 ml. of 50 per cent acetic acid in a centrifuge tube, and 1 ml. of the Griess Ilosvay reagent added immediately afterwards. The reagent was prepared fresh every day by mixing equal volumes of the following solutions: (*a*) 0·5 g. sulphanilic acid in 150 ml. 10 per cent acetic acid; (*b*) 1·0 g. *a*-naphthylamine in 180 ml. 10 per cent acetic acid. The samples were then centrifuged rapidly to deposit the cells, and the supernatant removed for comparison with simultaneously prepared standards in a Bausch & Lomb colorimeter. The colour reached a maximum in 30 min. and was stable for at least 48 hours. The standards were prepared from a stock solution of M/1 sod. nitrite kept at 4° and diluted freshly each time. The final concentration of nitrite in the three standard samples employed were respectively 4–, 10– and 20 $\times$ 10^{-5} M. Results were always recorded as concentration of nitrite (in terms of 10^{-5} M) present in the suspension-mixture. It was found that removal of the cells by centrifuge after adding the reagent and during development of the colour was the most satisfactory method of separation. If the cells were removed by centrifugation before addition of the reagent, rather low values were encountered—presumably due to the instability of the free HNO_2.

Estimation of tetrathionate reduction.—Results were expressed as ml. of N/200 I_2 per ml. of suspension-mixture. Pure sodium tetrathionate at a concentration of M/50 was used. For details see Pollock and Knox (1943).

Estimation of bacterial N.—The modification of the micro-Kjeldahl method described by Ma and Zuazaga (1942) was used. All estimations were done in duplicate. 2 ml. samples from the suspension mixture were added to 5 ml. of ice-cold distilled water in a centrifuge tube standing in iced water; after cooling, the cells were centrifuged down, further washed in 8 ml. of distilled water, and finally resuspended in 1 ml. of water before estimation.

Viable counts.—The method used was based on the surface drop technique of Miles and Misra (1938). Duplicate 0·5 ml. samples were diluted in 0·85 per cent saline in decimal series, and 6 separate drops (standardized at 41·7 drops per ml.) from each suitable dilution were dropped on to each of a pair of specially dried agar plates. These were incubated at 37° for 15 hours, and those plates giving between 75 and 200 colonies used for counting. Thus 4 plates and a minimum of 300 colonies were counted for each separate enumeration.

Turbidity measurements.—The relative opacities of suspension-mixtures before and after adaptation were estimated on the Hilger Spekker absorptiometer using a neutral grey filter (H 508). Duplicate 0·5 ml. samples were suitably diluted (usually 1 : 20) in ice-cold water, and estimated directly without further treatment. Results were recorded arbitrarily as the actual drum readings.

RESULTS.

Adaptation without growth.

In the case of tetrathionase of *Salm. paratyphi B*, it was shown (Knox and Pollock, 1944) that adaptation would occur with washed suspensions without additional source of N or change in the viable or total counts, dry weight of bacteria or cellular N. Similarly, with nitratase of "1433," adaptation was found to occur in the presence of mannitol, KNO_3 and buffer, there being a minimum of a 5-fold increase in activity of nitratase over a period of 2–4 hours, and there was no detectable change in the number of viable organisms, opacity measurements or bacterial N before and after adaptation (Table I).

Adaptation was allowed to proceed for up to 4 hours, after which the cells were centrifuged down, washed with distilled water and resuspended in water to a concentration usually 1/10 or 1/20 of the original suspension. These adapted cells were then found to reduce nitrate in the presence of a suitable H-donator at a greatly increased and approximately constant velocity. There was little falling off in the activity of these suspensions for several weeks if kept at 4°.

TABLE I.—*Nitratase Adaptation without Cell Growth.*

Viable counts, bacterial N, opacity measurements and nitratase activities of suspensions of "1433" before and after adaptation to nitrate at 35°, with M/50 mannitol, M/50 nitrate and M/5 buffer.

Expt. No.	Duration of expt.	Estimation.	Before adaptation.	After adaptation.
1	3½ hours	Viable count	$7{\cdot}05\times10^{8}$/ml.	$6{\cdot}39\times10^{8}$/ml.
		Opacity (drum reading after diluting 1 : 2)	0·304	0·292
		Nitratase activity: conc. of nitrite formed in $M\times10^{-5}$ per hour at 35° with mannitol	1·1	28·0
2	2¼ „	Viable count	$11{\cdot}9\times10^{9}$/ml.	$11{\cdot}5\times10^{9}$/ml.
		Bacterial N	0·33 mg./ml.	0·31 mg./ml.
		Opacity (drum reading after diluting 1 : 20)	0·376	0·386
		Nitratase activity: conc. nitrite formed in $M\times10^{-5}$ per hour at 35° with formate	17·5	110

A comparison of nitrate reduction by the same suspension at the same concentration of cells during and after adaptation is shown in Fig. 1.

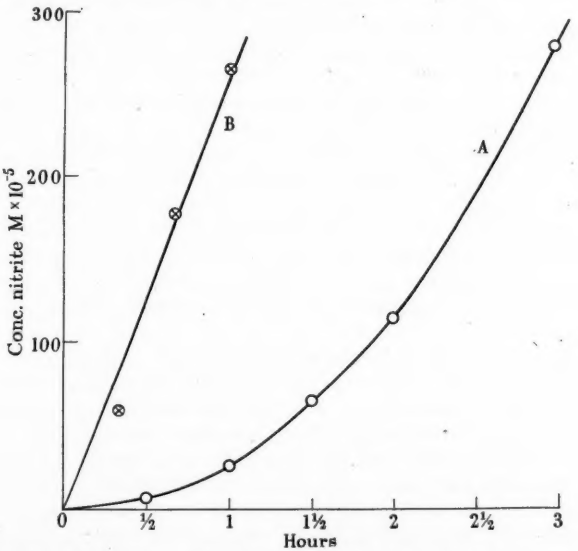

FIG. 1.—Adaptation of nitratase. Nitrate reduction by suspension of "1433" at 37° in M/50 KNO_3, M/50 mannitol and M/5 phosphate buffer. A, Untreated suspension. B, Same suspension after 4 hours' adaptation in above mixture and subsequent washing.

With nitratase adaptation it is not, of course, possible to eliminate an external source of N, but it seems doubtful whether nitrate serves as a satisfactory source of N for growth of these organisms. Braun and Goldschmidt (1928) found

that *Salm. paratyphi B* would grow on $NaNO_3$ as source of N, but *Bact. coli* would not grow in repeated subculture on a nitrate-glucose-salt medium. Kisch (1918) found that some strains of some Salmonellas would grow on nitrate as sole source of N, and that a single strain of *Bact. coli* grew rather feebly. Growth of " 1433 " has very occasionally been obtained, using a large inoculum, in a medium containing only mannitol and salts with M/50 KNO_3 as sole N source. The variable results are perhaps what might be expected if the nitrate had first to be reduced *via* nitrite to ammonia or some easily assimilable form of N before growth could commence. In any case, it is unlikely that these considerations are relevant to the adaptation of nitratase over a period of only 2–4 hours.

Factors influencing the rate of nitrate reduction and adaptation of nitratase.

(1) *Nitrite.*—Estimation of nitrite concentration in the suspension-mixture has been used to follow the reduction of nitrate to nitrite, although it is known that, with *Bact. coli* using H_2 as H-donator, the further reduction of nitrite to NH_3 may be rapid (Woods, 1938). It was at first thought that this destruction of nitrite might prove to be a source of considerable error, but it was found that with " 1433," the rate of disappearance was relatively slow—never more than 10 per cent of the rate of nitrite production. Almost complete (80 per cent or more) reduction of M/50 nitrate to nitrite could be obtained by continued incubation. Moreover, there was no difference in the rates of nitrite destruction by nitrate-adapted and unadapted cells. This further metabolism of nitrite is admittedly the cause of slight inaccuracy in all recorded results, but it is unlikely that it could ever materially affect any of the conclusions, and it has for the present purposes been ignored.

Sodium nitrite is known to be inhibitory to the growth of *Bact. coli* at a concentration of about M/20 or more (Quastel, Stephenson and Whetham, 1925). Concentrations of nitrite up to M/50 were encountered towards the end of the adaptation process in some of our experiments, though in experiments with adapted cells the concentration did not usually exceed M/1000. Experiments showed that M/1000 nitrite had no inhibitory effect on nitrate reduction by adapted cells, and only slight and possibly insignificant inhibitory effect on the adaptation process.

(2) *Nitrate.*—No decrease in the speed of nitrate reduction by adapted cells occurred until the nitrate concentration fell below M/500—i.e. until the reaction, with an initial nitrate concentration of M/50, was 90 per cent complete.

(3) *Oxygen.*—Stickland (1931) found that nitratase was very sensitive to O_2, being 94 per cent inhibited by 20·9 per cent (atmospheric) O_2. All recorded experiments were therefore done in evacuated Thünberg tubes. It was found, however, that in practice, using concentrated suspensions, these anaërobic conditions made very little difference (as with tetrathionase, Pollock and Knox, 1943), the effect being to abolish a very small lag in nitrate reduction, due presumably to inhibition by dissolved O_2. The rate of O_2 diffusion into the suspension-mixture from the atmosphere is far slower than its consumption by the bacteria, and after this dissolved O_2 is used up, the rate of nitrate reduction is not greatly affected by O_2 tension above the liquid.

(4) *Buffer.*—All recorded experiments were done in M/5 phosphate buffer. No detectable change in pH occurred from beginning to end of any experiment.

This high concentration was used largely in order to allow direct comparison with tetrathionate reduction by the same organisms, during which there is a much greater tendency for the pH to drop because of the production of thiosulphuric acid. There was no difference between the rates of nitrate reduction by adapted cells with buffer concentrations of M/5 and M/50, but the higher concentration had a slightly inhibitory effect on the adaptation rate.

(5) *pH.*—Stickland (1931) found the optimum pH for nitrate reduction by *Bact. coli*, with lactate as H-donator, to lie between 6 and 7. Using adapted cells of " 1433 " and phosphate buffers of pH ranging between 6·4 and 7·9, results were obtained varying somewhat according to the H-donator used and also from one preparation of cells to another, although in all cases the activity began to fall when the pH rose above 7·2; but the total variations never amounted to more than 30 per cent. With the tetrathionase of " 1433 " it was found that the activity was still increasing with greater alkalinity at pH 7·8. In order therefore to study the two enzyme systems simultaneously in the same organism it was decided to do all experiments at pH 7·2, which is probably not far from the optima of both.

(6) *Growth medium.*—Rates of growth and final yields may be widely different on different media, and enzyme activities are known to vary with the growth phase reached as well as with the constituents of the medium; it is therefore difficult to make quantitative comparisons between media in relation to the nitratase activity or adaptability of the resulting suspension, without accurate knowledge of the relative rates of growth.

However, a suspension of " 1433 " prepared from the growth on the standard tryptic meat agar was compared with that derived from growth on (*a*) a 1 per cent peptone + meat infusion agar, and (*b*) a synthetic medium of the following composition: KH_2PO_4, 4·5 g.; NH_4Cl, 0·5 g.; $(NH_4)_2SO_4$, 0·5 g.; M/2 sod. lactate, 50 ml.; $FeSO_4$, 0·02 g.; $MgSO_4 . 7H_2O$, 0·02 g.; 2 per cent agar in water to 700 ml.; pH 7·6. The organisms were grown for 15 hours at 37° in all cases but the resulting growth yields were very different, being about 15 times heavier on the tryptic meat digest than on the synthetic medium. The suspensions were standardized to equal opacity, and nitrate reduction was followed with mannitol in the usual way (Fig. 2). Although the absolute rates of nitrate reduction differed considerably, the relevant fact is that ability to adapt existed in all suspensions, and was not found to be significantly different in cells grown with ammonia as sole source of N.

Anaërobic growth on tryptic meat digest agar resulted in suspensions markedly more active in initial nitrate reduction, but with relatively poor adaptive powers. This aspect of the problem was not, however, investigated further. Growth on tryptic meat digest agar containing 1 per cent nitrate produced, as might be expected, suspensions with an exceedingly active nitratase.

7. *Temperature.*—The tetrathionase of *Salm. paratyphi B* has been found to adapt most rapidly at a temperature of 35°–37°, with almost complete inhibition of adaptation at 44°, although enzyme activity itself increased with temperature up to 50° (Pollock, 1945). The tetrathionase of " 1433 " was found to adapt most rapidly at a slightly lower temperature (33°). No attempt has yet been made to establish an exact optimum temperature for adaptation of the nitratase of " 1433," but Fig. 3 shows the curves of nitrate reduction by " 1433 " (with mannitol as H-donator) at 37° and 44°, and demonstrates the same sort of inhibi-

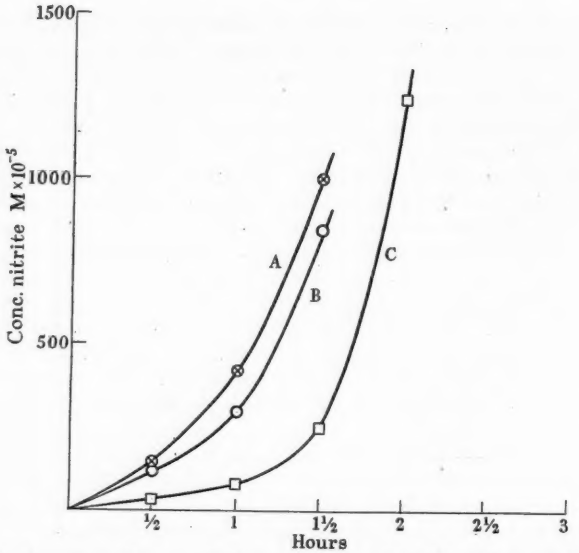

FIG. 2.—Effect of growth medium on nitratase adaptation of resulting cell suspensions. Nitrate reduction at 35° with mannitol by suspensions of " 1433 " grown on the following media : A, 1 per cent peptone plus meat infusion agar. B, Synthetic NH_3-lactate-salt agar. C, Tryptic meat agar.

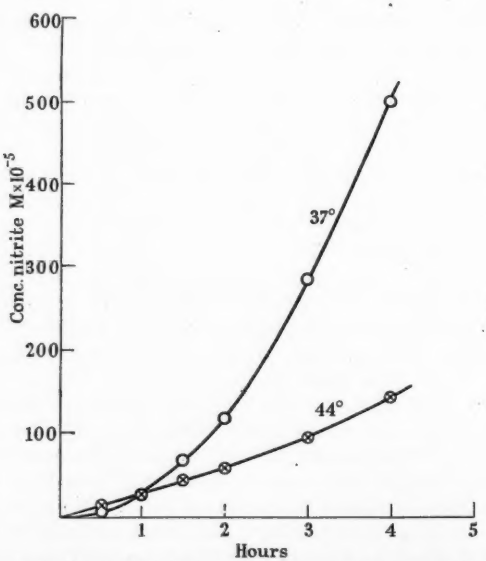

FIG. 3.—Nitratase adaptation at 37° and 44°. Comparison of nitrate reduction with mannitol by unadapted suspensions of " 1433 " at 37° and 44°.

tion of adaptation at 44° as that observed with the tetrathionase of *Salm. paratyphi B*. The activity of the nitratase itself—as studied in adapted suspensions—increased steadily with temperature up to 45°, after which there was little or no rise (Fig. 4), suggesting that it is inactivated at somewhat lower temperatures than the tetrathionase of *Salm. paratyphi B*.

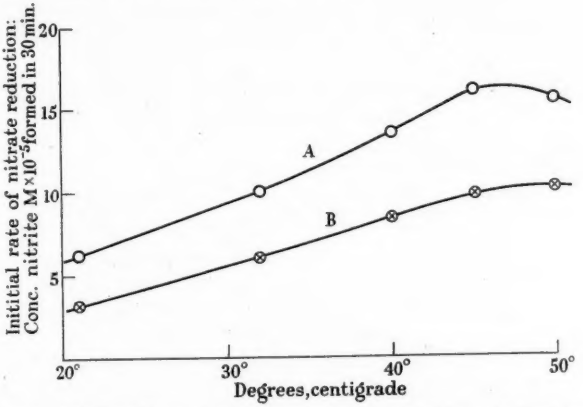

FIG. 4.—Influence of temperature on activity of nitratase of nitrate-adapted suspensions of "1433." A, Formate as H-donator. B, Mannitol as H-donator.

8. *H-donators*.—Mannitol, lactate and formate can all act as satisfactory H-donators for nitrate reduction using adapted cells. Table II shows the relative rates of the reaction with and without added H-donators. The "endogenous respiration" (in absence of H-donator) with dilute suspensions was always low and frequently undetectable. For adaptation, however, there was a great

TABLE II.—*Rates of Nitrate Reduction with Different H-donators at 35° by Nitrate-adapted Suspensions of* "1433."

H-donator.	Conc. nitrite formed in M × 10^{-5} per hour.
Formate	19·2
Mannitol	18·8
Lactate	18·2
No H-donator	*Nil*

difference between the three H-donators. Fig. 5 illustrates the reduction of nitrate by unadapted cells with and without different H-donators, and it can be seen that mannitol was the most efficient, lactate considerably less so, while with formate there was no apparent increase in nitratase activity at all. It has, in fact, been repeatedly confirmed that no adaptation occurs with formate as H-donator—or at least no more than the possibly very small amount which may take place during prolonged treatment with nitrate alone. Apart from its intrinsic interest this striking fact has been made use of in order to gauge more

accurately than is possible from the adaptation curves themselves the degree of adaptation that has occurred in any particular experiment.

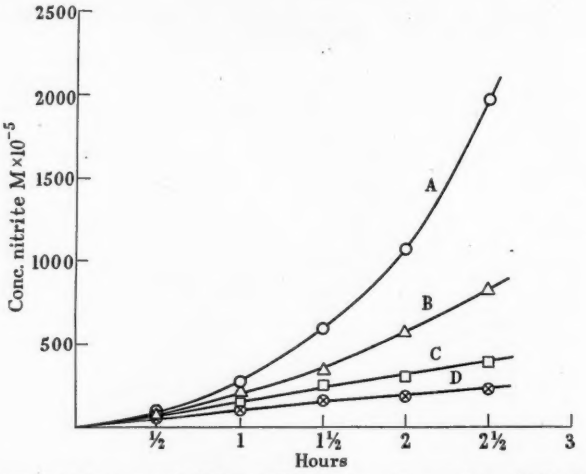

FIG. 5.—Nitratase adaptation with different H-donators. Comparison of nitrate reduction at 35° by unadapted suspension of " 1433 " using mannitol, sod. lactate, sod. formate as H-donators. A, Mannitol. B, Lactate. C, Formate. D, No H-donator.

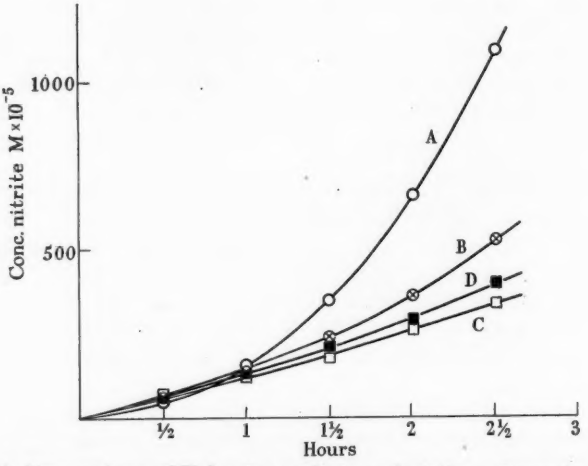

FIG. 6.—Effect of temperature and H-donator on nitratase adaptation. Comparison of nitrate reduction by unadapted suspensions of " 1433 " using mannitol and formate at 35° and 45°. A, Mannitol at 35°. B, Mannitol at 45°. C, Formate at 35°. D, Formate at 45°.

As previously pointed out (Pollock, 1945), adaptation curves such as those illustrated in Fig. 5 and 6 represent the combined effect of the particular set of

conditions upon both adaptation and the enzyme activity itself. This difficulty was previously overcome by removing samples, washing the cells and following the rate of their reduction of nitrate at 44°, at which temperature no adaptation occurred and all effects could be attributed to the action on the enzyme system (Pollock, 1945). The use of formate instead of mannitol for the purpose of measuring enzyme activity uncomplicated by adaptation is much more satisfactory, both because it can be followed at any temperature, and because use of another H-donator rules out the possibility of any increased nitratase activity

TABLE III.—*Effect of Previous Adaptation Treatment on Methylene-Blue Reduction by Suspensions of* " 1433."

Adaptation treatment (2½ hours at 35°).	Minutes for 90 per cent reduction of 1 : 25,000 methylene-blue at 35° with— (*a*) M/50 mannitol.	(*b*) M/50 formate.
Untreated control	9	11
Mannitol only (no H-acceptor)	24	15
Nitrate and mannitol	14½	14½
Tetrathionate and mannitol	25	16

being due merely to adaptation to mannitol. It might be argued, for instance, that adaptation to nitrate is really only a relative increase in the activity of an enzyme dehydrogenating mannitol. As a matter of fact there is independent evidence that this is not the case. In the first place, there is no increase but, in fact, a slight decrease in the activities of both mannitol and formate dehydrogenase, using the methylene-blue Thünberg tube technique for their demonstration, after adaptation (with mannitol) of either tetrathionase or nitratase (Table III). Secondly, as will be shown later, the adaptation process is strictly specific.

This method of demonstrating the adaptation effect is shown more clearly by the following experiment: Four aliquot portions of the same suspension were nitrate-adapted respectively with mannitol and formate at both 35° and 45°. Fig 6 shows that little adaptation occurred with formate at either temperature or with mannitol at 45°. After 2½ hours, samples of each suspension-mixture were removed, the cells washed, and subsequent nitrate reduction followed at 35° with formate. The relative nitratase activities (Table IV) show clearly the extent

TABLE IV.—*Relative Nitratase Activities of Suspensions of* " 1433 " *after Adaptive Nitrate Treatment under Different Conditions.*

Previous nitrate treatment (for 2½ hours).	Subsequent nitratase activity at 35° with formate as H-donator (conc. nitrite formed in M × 10^5 per hour).
Mannitol at 35°	90·5
,, 45°	22·2
Formate at 35°	18·1
,, 45°	16·7

of the adaptation that has occurred, and confirm the previous conclusion that both a satisfactory temperature and the correct H-donator are necessary for rapid adaptation.

The relative efficiencies of H-donators in adaptation are shown in a similar manner (Table V). It can be seen that there is 5 times more active enzyme after 2½ hours' treatment with mannitol compared with no H-donator or with formate, and 3 times more than with lactate.

TABLE V.—*Relative Nitratase Activities of Suspensions of "* 1433 *" after Adaptive Nitrate Treatment with Different H-donators.*

H-donator used in previous nitrate treatment (2½ hours at 35°).	Subsequent nitratase activity at 35° with formate as H-donator (conc. nitrite formed in M × 10^{-5} per hour).
Mannitol	81
Lactate	28·5
Formate	16·6
No H-donator	17·4

9. *Effect of dilution of suspensions.*—If the original unadapted suspension (at the usual strength) was diluted, say, 1 : 20, it was found that the rate of nitrate reduction per cell and the whole process of adaptation were very considerably slowed. Repeated washing of cells did not modify this effect. With adapted suspensions, the same phenomenon, though much less marked, was observed if mannitol was used as H-donator ; with formate, however, there was no dilution effect at all, the rate of the reaction being strictly proportional to the concentration of the suspension. Formerly (Yudkin, 1937) this disproportional falling off in an enzyme reaction has been used as evidence for the participation of a third component—probably a coenzyme. The question is of considerable interest, and will be dealt with more fully in a later report.

Specificity of adaptation.

Five equal portions of a suspension of " 1433 " were treated as follows :

(*a*) Untreated control.
(*b*) Mannitol and buffer at 35° for 2 hours.
(*c*) Mannitol, buffer and nitrate at 35° for 2 hours.
(*d*) Mannitol, buffer and tetrathionate at 35° for 2 hours.
(*e*) Mannitol, buffer, tetrathionate and nitrate at 35° for 2 hours.

The treated cells were centrifuged down after treatment, washed thoroughly with water, resuspended to their original concentration, and their activities and that of the control in reducing tetrathionate and nitrate followed at 35° with formate as H-donator (Table VI). It is quite clear that adaptation in each case has only occurred towards the particular substrate that was present, and that the process is quite specific, and can apparently take place without mutual interference between the two H-acceptors.

In a further experiment, three portions of a suspension of " 1433 " were doubly adapted to both nitrate and tetrathionate with mannitol at 35° in three different ways :

(*a*) 2¼ hours' treatment with nitrate followed by washing and 2¼ hours' treatment with tetrathionate.
(*b*) 2¼ hours' treatment with tetrathionate followed by washing and 2¼ hours' treatment with nitrate.
(*c*) 2¼ hours' simultaneous treatment with nitrate and tetrathionate.

TABLE VI.—*Specificity of Adaptation. Relative Tetrathionase and Nitratase Activities of Suspensions of "1433" Adapted to Tetrathionate and Nitrate Separately and Together.*

Previous adaptation (at 35° for 2 hours).	Subsequent reducing activities at 35° with formate as H-donator. (*a*) Nitratase (conc. nitrite formed in M × 10^{-5}/hr.).	(*b*) Tetrathionase (ml. N/200 I_2 per ml. per hour).
Untreated control	16·2	<0·1
Mannitol only (no H-acceptor)	16·7	<0·1
Nitrate and mannitol	61·6	<0·1
Tetrathionate and mannitol	15·8	4·1
Tetrathionate, nitrate and mannitol	59·2	5·25

The resulting suspensions, after thorough washing, were investigated for nitratase and tetrathionase activity, and compared with the original untreated suspension. In this case mannitol was used as H-donator. In such an experiment the conditions cannot be kept properly controlled and exact equivalence of results could hardly be expected, but Table VII shows that considerable

TABLE VII.—*Double Adaptation to Nitrate and Tetrathionate. Effect of Treatment of Suspensions of "1433" with H-acceptors Successively and Simultaneously.*

Adaptation treatment (with mannitol at 35° in all cases).	Subsequent reducing activity at 35° (mannitol as H-donator) (*a*) Nitratase (Conc. nitrite formed in M × 10^{-5} per hour).	(*b*) Tetrathionase (ml. N/200 I_2 per ml. per hour.)
2½ hours with tetrathionate followed by 2½ hours with nitrate	53·2	6·2
2½ hours with nitrate followed by 2½ hours with tetrathionate	17·4	4·4
2½ hours with tetrathionate and nitrate simultaneously	40·0	4·4
Untreated control	1·7	<0·1

double adaptation had occurred in each suspension, and there was no indication (as had been thought possible) that the most efficient adaptation had been towards that substrate which had been presented to the cells first.

Nitratase adaptation by Bact. coli *and* Salm. paratyphi B.

Suspensions of *Bact. coli* and *Salm. paratyphi B* were tested, under exactly the same conditions, for nitratase adaptation. In neither case was adaptation detectable using formate as H-donator, but with mannitol the nitratase activity of *Bact. coli* increased 3-fold after 3 hours' incubation at 35°, and that of *Salm. paratyphi B* 6-fold after 2½ hours at 35°. Table VIII shows the increase in nitratase activity of *Salm. paratyphi B* that occurred with mannitol at 35° after 2½ hours without any change in bacterial N or turbidity. There seems to be no reason

TABLE VIII.—*Nitratase Adaptation Without Growth by Washed Suspension of Salm. Paratyphi B.*

	Before adaptation.	After 2½ hours' adaptation at 35° with nitrate and mannitol.
Bacterial N	0·39 mg./ml.	0·38 mg./ml.
Opacity (drum reading after diluting 1 : 20)	0·212	0·211
Nitratase activity at 35° with formate as H-donator; conc. nitrite formed in M × 10^{-5} per hour	4·4	25·8

for supposing that the phenomenon of adaptation is much different in *Salm. paratyphi B* from that in "1433."

DISCUSSION.

The adaptation of nitratase by washed suspensions of bacteria appears to be essentially similar to that of tetrathionase. There is, perhaps, less relative increase in nitratase under comparable conditions, but this may be due to an apparently greater initial activity in unadapted cells. The adaptation of bacterial enzymes in the absence of, or at least independently of cell growth is probably a phenomenon more widespread than is commonly realized. It is perhaps surprising that it has not previously been reported for an enzyme-system as well known as nitratase. In this connection it is interesting to refer again to Stickland's original paper (1931), where he claims that the rate of nitrate reduction by dilute washed suspensions of *Bact. coli* is constant for at least 90 minutes. This is not surprising, since under such conditions little or no adaptation might be expected to occur whatever H-donator is used. He goes on to point out, however, that with a thick suspension the whole course of the reduction of M/50 nitrate to nitrite can be followed over a period of 5 hours. The explanation of why the phenomenon of adaptation was missed is not far to seek—although a study of the published figure does actually show an upwardly curved graph indicating increased nitratase activity of about 100 per cent which is not commented upon. The reason is no doubt because lactate was used as the H-donator and the reaction followed at 40°. It has previously been pointed out that lactate is a relatively inefficient H-donator for adaptation, and, to judge from results obtained with "1433" and *Salm. paratyphi B*, 40° is almost certainly well above the optimum adaptation temperature. If the reaction had been followed at 35° with mannitol, it is most likely that the upward trend of the curve would have been too striking to have been ignored.

The enzyme-system nitratase, like tetrathionase, cannot of course yet be regarded as a single enzyme; it depends for its action upon the activation of H-donators by other enzymes as well as upon the interaction of a H-carrier. For these and for other reasons already pointed out (Knox and Pollock, 1944) it is impossible to claim that increased nitratase activity necessarily represents the production in the cell of a single enzyme protein. But whether the hypothetical enzyme protein is visualized as being synthesized *de novo* or merely resulting from specific alteration of some indifferent cell protein (which is one of the suggestions

put forward by Spiegelman (1945) to explain enzyme adaptation), this still remains the most likely explanation. The complete specificity of tetrathionase and nitratase adaptation within the same cell suspension lends support to this hypothesis. If, for instance, nitratase adaptation depended largely upon coenzyme production or formation of a dehydrogenase rather than upon an enzyme specifically activating the reduction of nitrate, one might expect in addition some difference in the subsequent rate of reduction of tetrathionate which in almost every respect is a very similar process to reduction of nitrate. No such alteration occurs; cells adapted to nitrate reduce tetrathionate no more rapidly than untreated cells, and *vice versa*. Moreover, adaptation to either tetrathionate or nitrate in the presence of mannitol does not lead to any increased activity of mannitol dehydrogenase, as judged by the methylene-blue reduction test.

Both nitratase and tetrathionase adaptation curves are similar to those of the adaptation in washed suspensions of formic hydrogenlyase of *Bact. coli* (Stephenson and Stickland, 1932), yeast galactozymase (Schultz, Atkin and Frey, 1940; Spiegelman, 1945), and yeast glucozymase and melibiozymase (Spiegelman, 1945). In all these cases the beginning of adaptation is characterized by a rising rate of enzyme "formation" and the curves are not far removed from the exponential. It has been argued (Spiegelman, 1945) that this enzyme adaptation is due to the self-reproduction of either the enzyme protein itself or some protein or nucleo-protein precursor. Some such explanation would certainly appear to be necessary in order to understand the apparent self-perpetuation of the melibiozymase of haploid progeny of *S. cerevisiae–S. carlsbergensis* hybrids in the absence of the gene known to be originally responsible for its production (Spiegelman, Lindegren and Lindegren, 1945). The genetical approach to the problem has been considered in some detail by Spiegelman (1945) and Lindegren (1945). Lindegren (1945) goes so far as to equate the adaptive enzyme to his "cytogene," which he considers to be a self-reproducing cytoplasmic entity differing only from Darlington's "plasmagene" in being dependent upon the presence of a specific gene for its perpetuation in the absence of the specific substrate. Spiegelman and Lindegren's work has been done on nucleated yeast cells where genetical factors can be exactly determined, but these hypotheses, particularly in the case of bacteria, remain at present largely speculative.

SUMMARY.

1. Washed suspensions of a lactose-fermenting coliform organism ("1433") if incubated with mannitol and nitrate at 35° show a gradually increasing rate of reduction of nitrate to nitrite over a period of 2 to 4 hours.

2. The increase in nitratase activity, which may be up to 20 times (or more) that of the untreated suspension, is retained after repeated washing, and takes place without any increase in bacterial N, opacity measurements, or number of viable organisms.

3. Mannitol, lactate and formate can act as efficient H-donators for nitrate reduction by adapted cells, but no adaptation will occur with formate, and only a little with lactate. Adaptation with mannitol is by far the most rapid.

4. Little or no adaptation occurs at 44° or above.

5. Adaptation of nitratase appears to be closely analogous to that of tetrathionase.

6. Nitratase adaptation in washed suspensions is specific to nitrate and will not occur in its absence. Adaptation to both nitrate and tetrathionate can be induced independently in the same bacterial suspension by simultaneous or successive treatment with the two substrates.

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INDEX TO AUTHORS

ALLSOPP, C. B., see *Fell, H. B.*

ANDERSON, S. G., see *Burnet, F. M.*

ARMSTRONG, E. C., and BONSER, G. M., re-testing of a strain (IF) of mice specially sensitive to carcinogenic agents applied to the skin, 97

ARNSTEIN, H. R. V., COOK, A. H., and LACEY, M. S., the production of antibiotics by fungi. Part II: Production by *Fusarium javanicum* and other *Fusaria*, 349

BAWDEN, F. C., and PIRIE, N. W., the virus content of plants suffering from tobacco mosaic, 81

BECK, STEPHAN, the effect of feeding carcinogenic hydrocarbons dissolved in aqueous soap solution on the stomach of CBA mice, 155

BIELSCHOWSKY, F., tumours produced by 2-anthramine, 54

——— comparison of the tumours produced by 2-acetyl-amino-fluorene in piebald and wistar rats, 135

BONSER, G. M., see *Armstrong, E. C.*

BOWLER, R. G., see *Mather, K.*

BUECHLER, E., see *Olitzki, L.*

BURNET, F. M., MCCREA, J. F., and STONE, J. D., modification of human red cells by virus action. I. The receptor gradient for virus action in human red cells, 228

——— and ANDERSON, S. G., modification of human red cells by virus action. II. Agglutination of modified human red cells by sera from cases of infectious mononucleosis, 236

——— modification of human red cells by virus action. III. A sensitive test for mumps antibody in human serum by the agglutination of human red cells coated with a virus antigen, 244

CARR, J. G., the tumour virus disseminated from filterable to non-filterable tumours, 1

COLLUMBINE, H., and RYDON, H. N., a study of the formation, properties and partial purification of leukotaxine, 33

COOK, A. H., see *Arnstein, H. R. V.*

CROOK, E. M., and SHEFFIELD, F. M. L., electron-microscopy of viruses: I. State of aggregation of tobacco mosaic virus, 328

CROOKE, A. C., see *Mather, K.*

DAFF, M. E., see *Kennaway, E. L.*

DICKER, S. E., HELLER, H., and HEWER, T. F., renal effects of protein-deficient vegetable diets: a functional and histological study, 158

DMOCHOWSKI, L., the preservation of mammary tumour agent by desiccation of the breast tumour tissue of mice, 391

FELL, H. B., and ALLSOPP, C. B., the effects of lewisite and of lewisite oxide on living cells *in vitro*, 305

——— the therapeutic effect of British anti-lewisite (BAL) on tissue cultures grown in a medium containing lewisite oxide, 310

FLOREY, H. W., see *Heatley, N. G.*

FULTON, J. D., see *Harrison, C. V.*

GELL, P. G. H., HARINGTON, C. R., and RIVERS, R. P., the antigenic function of simple chemical compounds: production of precipitins in rabbits, 267

GLADSTONE, G. P., immunity to anthrax: protective antigen present in cell-free culture filtrates, 394

GRIESBACH, W. E., see *Purves, H. D.*

HARINGTON, C. R., see *Gell, P. G. H.*

HARPER, G. J., see *Williams, R. E. O.*

HARRISON, C. V., and FULTON, J. D., the effect of treatment on the spleen of the golden hamster (*Cricetus auratus*) infected with *Leishmania donovani*, 4

HEATLEY, N. G., and FLOREY, H. W., an antibiotic from *Bacterium coli*, 378

HELLER, H., see *Dicker, S. E.*

HERBERT, S., see *Selbie, F. R.*

HEWER, T. F., see *Dicker, S. E.*

HOCH, H., and HOCH, R., the state of vitamin A in human serum, 316

HOCH-LIGETI, C., the effect of the administration of *p*-dimethylaminozobenzene on the ovarian functions of rats, 211

JENKINS, C. E., the release of antibody by sensitized antigens, 111

——— the preparation of pure flagellar antigens from Salmonella, 121

——— a study of the prozones of the Salmonella, 127

KELLAWAY, E. L., and DAFF, M. E., the effect of diet upon the ascorbic acid content of the liver in mice, 63

KIRBY, A. H. M., and PEACOCK, P. R., the influence of methylation on carcinogenic activity. I. N-methyl-3 : 4 : 5 : 6-dibenzcarbazole, 179

LACEY, M. S., see *Arnstein, H. R. V.*

MACFARLANE, M. G., and SPOONER, S. J. L., chemical changes in muscle during and after ischaemia, 339

MACLAGAN, N. F., faecal urobilinogen : clinical evaluation of a simplified method of estimation, 190

——— the preparation and use of colloidal gold sols as diagnostic agents, 369

MARTIN, N. H., the components of the serum proteins in infective hepatitis and in homologous serum jaundice (an electrophoretic study), 363

MAYER-GROSS, W., and WALKER, J. W., taste and selection of food in hypoglycaemia, 297

MCCREA, J. F., see *Burnet, F. M.*

MCINTOSH, J., and SELBIE, F. R., further observations on the chemotherapy of experimental gas gangrene : flavazole, marfanil, V187 and V335, 46

MCQUILLAN, M. T., and TRIKOJUS, V. M., the thyrotrophic hormone and thiourea in reference to the problem of "antihormones," 247

MEDAWAR, P. B., immunity to homologous grafted skin. I. The suppression of cell division in grafts transplanted to immunized animals, 9

——— immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin, 15

MILES, J. A. R., a serological investigation in hepatitis using the complement-fixation reaction, 25

MORGAN, W. T. J., and SCHÜTZE, H., non-agglutinating antibody in human antisera to *Sh. shiga* and *S. typhi*, 286

MORRIS, C. J. O. R., see *Mather, K.*

MURRAY, J., the incidence of RH types in the British population, 102

OLITZKI, L., and BUECHLER, E., studies on the detoxication of the labile rickettsia toxin, 220

OSTER, G., and STANLEY, W. M., an electron microscope study of the contents of hair cells from leaves diseased with tobacco mosaic virus, 261

PEACOCK, P. R., see *Kirby, A. H. M.*

PINNIGER, J. L., and PRUNTY, F. T. G., some observations on the blood-clotting mechanism. The role of fibrinogen and platelets, with reference to a case of congenital afibrinogenaemia, 200

PIRIE, N. W., see *Bawden, F. C.*

POLLOCK, M. R., adaptation of "nitratase" in washed suspensions of bacteria, 419

PRUNTY, F. T. G., see *Pinniger, J. L.*

PURVES, H. D., and GRIESBACH, W. E., observations on the acidophil cell changes in the pituitary in thyroxine deficiency states. I. Acidophil degranulation in relation to goitrogenic agents and extrathyroidal thyroxine synthesis, 170

——— studies on experimental goitre. VII : Thyroid carcinomata in rats treated with thiourea, 294

RIVERS, R. P., see *Gell, P. G. H.*

RYDON, H. N., see *Collumbine, H.*

SCHÜTZE, H., see *Morgan, W. T. J.*

SELBIE, F. R., see *McIntosh, J.*

——— Shope papilloma and sheep dermatitis in the rabbit : mutual interference, superinfection, and effects of chemical pretreatment of the skin, 143

——— and HERBERT, S., effect of penicillin combined with neo-arsphenamine on the lesions and serological titre of experimental syphilis, 91

SHAPIRO, B., and WERTHEIMER, E., spontaneous crystallization of a protein from pathological human serum, 225

SHEFFIELD, F. M. L., see *Crook, E. M.*

SPOONER, S. J. L., see *Macfarlane, M. G.*

STANLEY, W. M., see *Oster, G.*

STONE, J. D., see *Burnet, F. M.*

TRIKOJUS, V. M., see *McQuillan, M. T.*

WALKER, J. W., see *Mayer-Gross, W.*

WERTHEIMER, E., see *Shapiro, B.*

WHITE, P. B., note on a variant of *B. anthracis* which fails to form heat-resisting endospores and on attempts to immunize guinea-pigs against anthrax infection with killed vaccines prepared from it, 356

WILKINS, W. H., investigation into the production of bacteriostatic substances by fungi. Preliminary examination of the fifth 100 species, all basidiomycetes, mostly of the wood-destroying type, 140

WILLIAMS, R. E. O., and HARPER, G. J., determination of coagulase and alpha-haemolysin production by staphylococci, 72